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Investigating the mechanisms mediating the outcomes of prenatal stress

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Declaration

I declare that all work produced in this thesis is my own, except in cases of collaborative work, which is clearly stated in the text and appropriate credit has been given. The work in this thesis has not been submitted, in whole or in part, in any previous application for a degree.

Part of the work presented in Chapter 3, attached as Appendix B, was published in the Journal of Neuroendocrinology as “Sex-dependent changes in neuroactive steroid concentrations in the rat brain following acute swim stress” by Ying Sze, Dr Andrew C Gill (co-supervisor), and Dr Paula J Brunton (primary supervisor). This study was conceived by all of the authors, whilst the experimental work and data analysis was carried out by myself. Assistance received from Dr Yu-Ting Lai and Mrs Helen Cameron in sample collection have been acknowledged in the published text.

Part of the data presented in this Chapter 6 was obtained in an experiment carried out by myself, Dr Paula J Brunton, Dr Tom J Phillips (Bristol) and Dr Hannah Scott (Bristol) at the Roslin Institute and the University of Bristol. The specific contributions from colleagues in the collaboration are explicitly stated in the text. I played a major role in the preparation and execution of the experiment, and the data analysis and interpretation in this thesis are entirely my own work.

Part of the work presented in Chapter 5 was carried out in collaboration with MSc student Ms Joana Fernandes, and work in Chapter 4 was carried out with the assistance of PhD student Ms Daniela Schnitzler. The specific contributions by these individuals have also been described in each of the chapters.

Signed:

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Abstract

Excessive stress during pregnancy can strongly impact the developing offspring, leading to long-term changes in the brain that persist into adulthood, in a process known as “prenatal programming”. In a rat model, where pregnant dams were exposed to five days of social stress from gestational day (GD) 16 to 20, the prenatally stressed (PNS) offspring exhibit anxious behaviour, cognitive deficits and exaggerated hypothalamic-pituitary-adrenal (HPA) axis responses to stress, with outcomes often dependent on the sex of the offspring. However, the mechanisms mediating these outcomes are not completely understood. Firstly, it is not completely clear what underlies the “programmed” phenotypes in adulthood, and secondly, it is not known how the stress signals are transmitted from the mother to the foetus during gestation to result in foetal “programming”.

Steroids are a large class of signalling molecules produced by endocrine organs, and include the glucocorticoids (e.g. corticosterone), sex hormones (e.g. progesterone and testosterone) and neuroactive steroids (such as allopregnanolone). Steroids play key roles in modulating stress responses, and are also involved in coordinating pregnancy adaptations and foetal development, therefore may be a common factor in the mechanisms underlying the “programmed” phenotypes observed in PNS offspring, and in the process of foetal “programming” during pregnancy. In order to investigate a role for steroids in foetal programming, it was first necessary to develop and validate a liquid chromatography-mass spectrometry method (LC-MS) that could reliably quantify a panel of steroids in complex tissues such as the brain.

Neuroactive steroids are known to increase following an acute stressor and can rapidly modulate neuronal excitability. It was hypothesised that alterations in neuroactive steroid concentrations in the brain may underlie the “programmed” phenotype, such as dysregulated HPA axis activity, in adult PNS offspring. Using the LC-MS method developed, no obvious differences were found in central neuroactive steroid concentrations between control and PNS offspring at baseline; however, following an acute stressor (swim stress), the production of certain neuroactive steroids which modulate inhibitory neurotransmission seem compromised in the brains of PNS offspring as compared to controls, which could be further investigated with other acute stress paradigms.

The possible mechanisms involved in the transmission of stress signals from the mother to foetus during gestation to result in “programming” were then investigated at GD20, focusing on glucocorticoid metabolism and the steroidal milieu in the mother and the foetus. Glucocorticoid overexposure has been proposed as one of the primary mechanisms of foetal programming. However, it was found that despite stressed dams having greater circulating corticosterone, stressed foetuses did not exhibit greater corticosterone concentrations in the brain. The steroidogenic profile was also generally similar in the brains of control and stressed foetuses. The direct contribution of altered corticosterone and neuroactive steroid concentration in “programming” of the foetal brain, and possibly behaviour, is therefore minimal. However, stressed placentae, exhibited greater mRNA expression for 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2), which inactivates corticosterone, indicating that the placenta plays an active role in regulating the transmission of gestational stress signals from the mother to the foetus during late pregnancy.

The role of the placenta was then further investigated, where it was hypothesised that placental oxidative stress may play a role in foetal programming. Elevated oxidative stress status was observed in the placentae of stressed pregnancies at GD20, which was prevented by the administration of an antioxidant drug to the pregnant dam before the onset of chronic social stress. Notably, this maternal antioxidant treatment, which targets the maternal compartment and placenta but does not reach the foetus, rescued the anxiety phenotype of PNS offspring when examined in adulthood. Several other physiological markers in the brain linked to anxiety and inhibitory neurotransmission, which were altered in PNS offspring as compared to controls, were also normalised as a result of the maternal antioxidant treatment, and the effects were sex specific. Additionally, both secretions from placental explants and foetal plasma from stressed pregnancies contained unknown factors that altered neuronal growth *in vitro*, but this was not observed in stressed pregnancies with antioxidant treatment. Together, this suggests that the stressed placenta may transmit stress signals to the foetus through secreting, as yet unidentified, damaging “factors”. Hence, targeting placental oxidative stress during pregnancy could provide a therapeutic option to prevent the transmission of such signals, and therefore the adverse outcomes observed in the offspring later in life.

Lay Summary

Stress that is experienced by the pregnant mother during pregnancy, can affect the growth and development of the unborn offspring, leading to enduring changes that are still observable in the offspring during adulthood. These may include an increased risk for mental illnesses such as anxiety, and a compromised ability for the offspring to cope when faced with stress themselves. However, how maternal stress may lead to these outcomes is not completely understood. Firstly, we do not fully understand how the behaviours in the adult offspring arise. Secondly, we do not know how the stress, which is experienced by the mother, is being transmitted to the developing foetus to result in these changes. In order to better understand these processes, a rat model was used, where pregnant rats were stressed by exposing them to another aggressive rat, to induce social stress and better reflect the type of stress experienced by pregnant women.

Steroid hormones play an important role in helping an organism cope with stress and are also crucial for maintenance of the pregnancy and ensuring proper growth of the foetus. As steroid hormones may be an important mechanism, a biochemical assay that is able to quantify multiple steroids in the brain was first developed. To establish if steroid hormones can lead to poor outcomes in the adult prenatally stressed offspring, steroids in their brains were quantified, however, no obvious differences were found in brain steroid levels between stressed and non-stressed offspring.

Next the factors that are involved in the transmission of stress signals were investigated. High levels of the stress hormone, corticosterone, has been proposed to cross the placenta to the foetus and directly impact the foetal development in a harmful manner. Although the stress hormone increased in the pregnant mother with stress, the placenta seemed to play a protective role, preventing the cross over of maternal stress hormones to the unborn foetus.

The placenta however, was also found to be undergoing oxidative stress (a type of stress on a cellular level, caused by imbalance between free radical and antioxidant production). In order to study the possible role of oxidative stress in prenatal programming, an antioxidant drug was given to the pregnant rat before it was exposed to stress, thereby blocking increases in oxidative stress. The antioxidant

drug given to the mother prevented the anxious behaviour in the stressed offspring during adulthood. Oxidative stress could therefore be involved in the transmission of stress signals from mother to foetus, and antioxidant treatment to the mother could potentially help to prevent the harmful effects of stress during pregnancy.

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Abbreviations

%CV	% coefficient of variation
11-DHC	11-dehydrocorticosterone
11β-HSD1	11β-hydroxysteroid dehydrogenase isoform 1
11β-HSD2	11β-hydroxysteroid dehydrogenase isoform 2
^{35}S–UTP	uridine triphosphate, labelled with radioactive isotope of sulfur
3α-diol	3α-androstanediol
3α-HSD	3α-hydroxysteroid dehydrogenase
3β-diol	3β-androstanediol
3β-HSD	3β-hydroxysteroid dehydrogenase
5-HIAA	5-hydroxyindoleacetic acid
5-HT	serotonin (5-hydroxytryptamine)
ACTH	adrenocorticotropic hormone
ANOVA	Analysis of Variance
AR	androgen receptor
AUC	area under curve
AVP	arginine vasopressin
AVP-R1a	vasopressin receptor type 1a
BLA	basolateral amygdala
BMA	basomedial amygdala
BNST	bed nucleus of the stria terminalis
BRF	Biological Resource Facility
BSA	bovine serum albumin
C18	octadecyl bonded silica
CA	cornu Ammonis (subfields of hippocampus)
CBG	corticosteroid-binding globulin
cDNA	complementary DNA
CeA	central amygdala
CNS	central nervous system
cpm	counts per minute
CRH	corticotropin releasing factor
CRH-R1	corticotropin releasing factor receptor type 1
CRH-R2	corticotropin releasing factor receptor type 2
DCF	fluorescent 2',7'-dichlorofluorescein
DCFDA	2',7'-dichlorofluorescein diacetate
ddH2O	ultrapure water
DEPC	diethylpyrocarbonate
DHDOC	dihydrodeoxycorticosterone
DHEA	dehydroepiandrosterone
DHP	dihydroprogesterone
DHT	dihydrotestosterone
DMABC	4-dimethylamino benzoyl chloride
DnCl	dansyl chloride
DOC	deoxycorticosterone
DOHaD	developmental origins of health and disease

DTT	dithiothreitol
EIC	extracted ion chromatograms
EPM	elevated plus maze
ER	oestrogen receptor
ESI	electrospray ionisation
FA	formic acid
FC	frontal cortex
FKBP	FK506-binding proteins
FSH	follicular stimulating hormone
FST	forced swim test
GABA	γ-aminobutyric acid
GC-MS	gas chromatography-mass spectrometry
GD	gestational day
GluN1	NMDA receptor subunit 1
GR	glucocorticoid receptor
HMP	2-hydrazino-1-methylpyridine
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
HPLC	high performance liquid chromatography
HSP	heat shock protein
Icj	islands of Calleja
IHC	immunohistochemistry
IL-1β	Interleukin 1 beta
ISH	*in situ* hybridisation
ISTD	deuterated internal standard, LC-MS
IUGR	intrauterine growth restriction
IVC	individually ventilated cage
JZ	junctional zone
KO	knockout
LB	labyrinth zone
LC	liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
LDB	light-dark box
LOD	limit of detection
LOQ	limits of quantification
LS	lateral septum
m/z	mass-to-charge ratio
MAP2	microtubule-associated protein 2
MeA	medial amygdala
MeOH	methanol
MitoQ	mitoquinone
MitoQ-NP	mitoquinone (MitoQ) loaded in a nanoparticle delivery system
mPFC	medial prefrontal cortex
mPOA	medial preoptic area
mpPVN	medial parvocellular part of the paraventricular nucleus
MR	mineralocorticoid receptor

MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MS1	mass spectrometry (scanning/non-fragmentation mode)
MS2	mass spectrometry (fragmentation mode)
NIH	National Institutes of Health
NMDA	N-methyl-d-aspartic acid
NP	nanoparticles
Nrf2	nuclear factor erythroid 2-related factor 2
NTS	nucleus of the solitary tract
OCT	optimal cutting temperature compound
OGT	O-linked N-acetylglucosamine transferase
p450scc	p450 side-chain cleavage enzyme
PBS	phosphate buffered saline
PFA	paraformaldehyde
PFC	prefrontal cortex
PND	postnatal day
PNS	prenatally stressed
POMC	pro-opiomelanocortin
PR	progesterone receptor
PTSD	post-traumatic stress disorder
PTSI	p-toluenesulfonyl isocyanate
PV	parvalbumin
PVN	paraventricular nucleus of the hypothalamus
QC	quality control
r^2	coefficients of determinant
RIA	radioimmunoassay
ROS	reactive oxygen species
RT	room temperature
SHBG	sex hormone-binding globulin
SNK	Student-Newman-Keuls
SNS	sympathetic nervous system
SOD	supraoxide dismutase, antioxidant enzyme
SPE	Solid-phase extraction
SSC	saline sodium citrate
StAR	steroidogenic acute regulatory protein
TFA	trifluoroacetic acid
TH	tyrosine hydroxylase
THDOC	tetrahydrodeoxycorticosterone
TIC	total ion chromatograms
TPP	lipophilic triphenylphosphonium

Chapter 1: GENERAL INTRODUCTION

1.1 Developmental Origins of Health and Disease

The concept of developmental origins of health and disease (DOHaD) was first developed in 1989 by Dr. David Barker and colleagues (Barker, 1990), following epidemiological observations that chronic diseases in adulthood, such as heart disease, hypertension, and stroke was correlated with placental and offspring birth weight, which can subsequently be traced back to adverse intrauterine conditions during foetal development. Since then, there has been a wealth of epidemiological, clinical, and experimental data supporting the notion that in addition to genetics, the early life environment also plays a crucial role in setting the stage for disease later in life. This phenomenon is referred to as “prenatal programming”, where there is a permanent change in the physiology of an organism following a stimulus or insult during a critical and sensitive period in early life (Godfrey and Barker, 2001).

While initial DOHaD studies focussed on the role of maternal undernutrition in foetal programming, the DOHaD approach has since been extended to studying the effects of foetal exposure to psychobiological-related factors such as maternal psychological stress, paternal stress, or even the transgenerational effects of grand-maternal stress (Matthews and Phillips, 2012). In terms of offspring outcomes, the persistent effects of prenatal programming have also been analysed not only from a physical health perspective, but also with regards to the offspring’s psychological health, for instance, their propensity to develop to stress-related disorders in adulthood. The DOHaD approach provides a framework in which stress-related disorders can be studied throughout an individual’s life course and across generations, and is extremely relevant today, given that mental health and behavioural problems are one of the main causes of the overall disease burden and disability worldwide (Vos and Global Burden of Disease Study, 2015).

There has been ample progress made in the past three decades to define the molecular pathways that may possibly underpin these associations, however, the multidimensional nature of DOHaD means that much more needs to be done in order to gain a greater mechanistic understanding of the underlying processes. This understanding would then allow the DOHaD concept to be of a greater significance not only in the clinic, but also in terms of shaping public health and global economic policy.

This chapter will first begin with defining what “stress” entails, highlighting basic biological and molecular concepts related to stress, with a focus on the hypothalamic-pituitary-adrenal (HPA) axis and steroids as a modulatory factor. Next, the physiological concepts related to a successful pregnancy are introduced, and the impacts of stress on the pregnancy and offspring outcomes during adulthood are discussed. There is a particular focus on rodent models, which are the basis of experiments carried out in this body of work. Finally, this chapter will briefly summarise current mechanistic understanding of how negative phenotypes in offspring behaviour may arise, and highlight some of the known or proposed mechanisms of stress transmission from the mother to the foetus. As the field is now extremely large and underlying processes of foetal programming have been revealed to be increasingly complex, it is virtually impossible to fully consider all aspects and all the possible mediators and regulators involved. This thesis will therefore focus on exploring the role of glucocorticoids, neuroactive steroids and oxidative stress in determining offspring behaviour and physiology.

1.2 What is stress?

To begin, an inquiry of what exactly constitutes “stress” first has to be carried out. Although the word “stress” seemingly carries negative connotations, it represents an important physiological adaptation that is essential for an organism’s survival following changes in the environment. The concept of “stress” was first conceived by Walter Cannon in the 1920s and describes the phenomena where there is disruption of the body’s equilibrium, leading to activation of the sympathetic nervous system (SNS) and the “fight-or-flight” response in order to reinstate homeostasis (Cannon, 1914). Hans Selye, in 1936, discovered when experimenting in rats that in addition to the activation of the SNS, several changes occur in the adrenal glands and immune system when the animals are exposed to various nocuous stimuli (Selye, 1936). These changes apply to all mammals and humans, and constitutes what is known today as the hypothalamic-pituitary-adrenal (HPA) axis (Miller, 2018, Szabo et al., 2012). Selye further recognised that “stress” can not only be acute but also can be chronic in nature, and defined it as a “non-specific response of the body to any demand for change” (Selye, 1950). In contemporary biology, most agree that “stress” can be defined as “a real or interpreted threat to the physiological or psychological integrity of an organism that results in physiological and/or behavioural responses” (McEwen, 2000). Stress constitutes the activation of both the SNS and the HPA axis (elaborated in section 1.2.1), resulting in changes in other neuroendocrine, autonomic, immune, and metabolic systems, which eventually allows the organism to cope with the stressful event (Chrousos and Gold, 1992). Therefore, stress in itself, is adaptive and necessary for the survival of the organism.

Although stress responses are adaptive in nature, Selye also recognised that prolonged or excessive activation of the neuroendocrine stress system can lead to disease, where “stress” then becomes pathological (Selye, 1950). Following observations from rats and human patients, Selye proposed that pathological forms of stress can arise from the production and action of excess glucocorticoids, leading to the exhaustion and failure of bodily systems (Selye, 1950). However, in recent decades, the complexity of the stress response has become apparent, and it is known that various environmental, genetic, psychological, immune or metabolic factors can interact and cause stress-related disorders (Chrousos and Gold, 1992, Schneiderman et al., 2005). To address this complexity, a novel concept called

“allostasis” initially proposed by Sterling and Eyer (Sterling and Eyer, 1988) was introduced by McEwen into the field of stress research (McEwen, 1998). “Allostasis” is a term used to describe the dynamic regulatory process that allows the organism to adapt to the challenges of its environment, and is also able to explain how stress-related pathologies can occur when these regulatory processes fail. Stress in the context of “allostasis” will be discussed in section 1.2.2, where focus will be placed largely on the HPA axis, as its dysregulation is one of the most consistent findings in stress-related disorders (e.g. in affective disorders such as depression) (Plotsky et al., 1998).

1.2.1 The classic stress response

The response to a prototypical acute stressor in humans and mammals involves activation of two systems, the SNS and the HPA axis, which then result in downstream physiological changes in various organs and systems to cope with the stressor. Some of the physiological changes include the mobilisation of the body's energy stores, increasing gluconeogenesis, cardiovascular tone and respiratory rate, whilst digestive, reproductive and immune systems are inhibited. Behavioural adaptations also occur, which includes increased alertness and attention, improved cognition, and also central inhibition of appetite, feeding, and reproduction (Thiel and Dretsch, 2011, Tsigos et al., 2000).

1.2.1.1 Sympathetic nervous system

Upon experiencing stressful stimuli, the SNS is first engaged, where the brainstem is activated, leading to activation of preganglionic fibres in the spinal cord. Preganglionic fibres innervate the adrenal medulla and directly activates it via cholinergic signals, leading to the release of adrenaline and noradrenaline into the circulation (Fig 1.1). At the same time, preganglionic fibres innervate the ganglion, leading to postganglionic fibres releasing noradrenaline locally in target organs, such as those in the cardiovascular, musculo-skeletal, and digestive system. The stress-induced release of noradrenaline and adrenaline from the SNS peaks within 1-5 min, and together, these catecholamines act on α- and β-adrenergic receptors throughout the body, bringing about various physiological effects such as increased heart rate, dilation of blood vessels in skeletal muscle and increased glycogen-to-glucose conversion. Reduced blood flow and vasoconstriction of blood vessels also occur in the digestive and reproductive organs. In the brain, activation of the

brainstem, specifically the locus coeruleus, which has dense noradrenergic neurones, also leads to activation of a widespread noradrenergic neuronal network throughout the brain, resulting in increased arousal (Aston-Jones et al., 1986). The activation of the SNS is counter-controlled by the parasympathetic system, which dampens the effect of the SNS when its activation is no longer required (Ulrich-Lai and Herman, 2009).

1.2.1.2 Hypothalamic-Pituitary-Adrenal (HPA) Axis

The second arm of the stress response, the HPA axis, is also activated but usually after a short delay relative to SNS activation. Upon stimulation by stress, neurones in the medial parvocellular part of the paraventricular nucleus (mpPVN) of the hypothalamus release the peptides corticotropin releasing factor (CRH) and arginine vasopressin (AVP) into the hypophyseal portal system, which then enters the anterior pituitary gland. In corticotroph cells of the anterior pituitary, another peptide, adrenocorticotropic hormone (ACTH), is synthesised from the cleavage of the precursor pro-opiomelanocortin (POMC) and is then released by the anterior pituitary into the general circulation. ACTH then stimulates the adrenal cortex to increase steroid production in the adrenal gland and release glucocorticoids into the bloodstream (cortisol in humans and corticosterone in rats and mice) (Fig 1.1).

Glucocorticoids travel in the bloodstream and have pleiotropic effects at multiple target organs and tissues, via their action on the glucocorticoid receptor (GR; *Nr3c1*) and mineralocorticoid receptor (MR; *Nr3c2)*. Their primary effects are the redistribution of energy across the body in the face of stressor, promoting processes related to energy mobilisation in the brain and skeletal muscles, but inhibiting processes related to growth, development, reproduction and immunity (Sapolsky et al., 2000, Munck et al., 1984). Given the widespread effects of glucocorticoids and that GR and MR are ubiquitously expressed in all cell types and tissues, prolonged elevation of circulatory glucocorticoids can lead to deleterious effects and its production needs to be tightly controlled. The glucocorticoid response curve has a rapid onset, peaking at about 15-30 min, and then gradually decreasing with time from inhibitory mechanisms that shut it down (Herman, 2013).

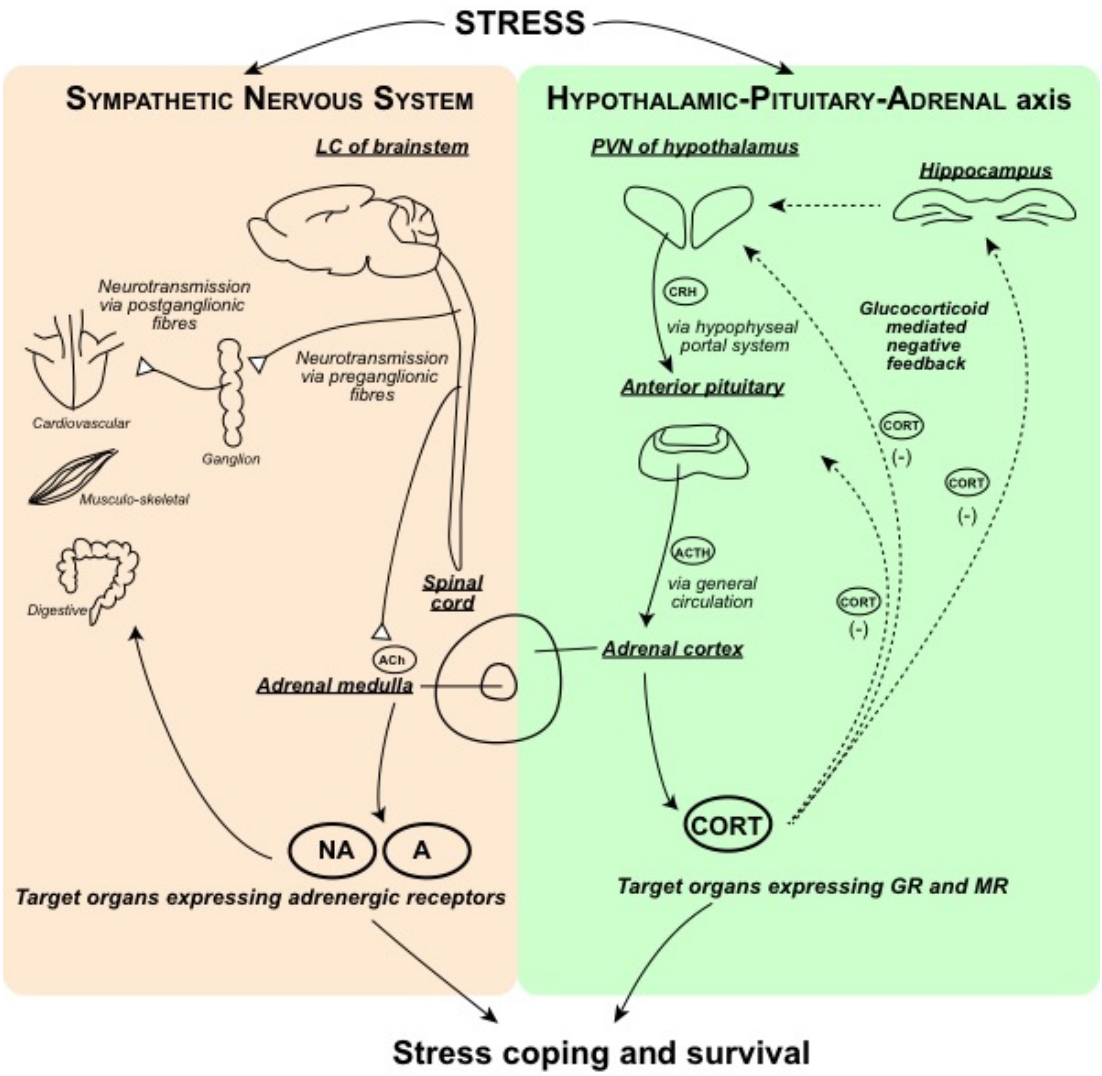


Figure 1.1: Two arms of the prototypical stress response – the SNS and the HPA axis. Both result in the activation of the adrenal gland, which plays a central role in the stress response. The adrenal cortex produces and secretes cortisol/corticosterone (CORT), in humans/rats respectively, into the bloodstream following activation of the HPA axis, while the adrenal medulla produces noradrenaline (NA) and adrenaline (A) following activation by the SNS. The HPA axis is self-limiting, where glucocorticoids can act via negative feedback inhibition, while the sympathetic nervous system is counter-acted by the parasympathetic nervous system. Abbreviations: NA: noradrenaline; A: adrenaline; CORT: corticosterone (in rats); cortisol (in humans), MR: mineralocorticoid receptor; GR: glucocorticoid receptor; PVN: paraventricular nucleus of the hypothalamus; LC: locus coeruleus of the brainstem. Illustrations in the figure are representative of the rat anatomy.

1.2.1.3 Regulation of the HPA axis

There exist complex mechanisms in the brain that orchestrate the activation and inhibition of the HPA axis. One of the major inhibitory mechanisms of the HPA axis is negative feedback control mediated by glucocorticoids, which act on GR receptors in the hippocampus, PVN, and anterior pituitary to terminate the HPA axis response. Different signals originating from different parts of the brain are ultimately integrated at the PVN to result in the appropriate secretion of CRH (Herman et al., 2016, Herman et al., 2002, Smith and Vale, 2006). In general, neural communication between brain regions are regulated by glutamate and γ-aminobutyric acid (GABA) action on ligand-activated ion channel receptors, where glutamate generally elicits an excitatory action while GABA exhibits an inhibitory action. Brain regions that are involved in the modulation of the HPA axis include not only the hypothalamus and the pituitary gland, but also regions of the limbic system (consisting of the prefrontal cortex, hippocampus and amygdala), which are responsible for the generation of appropriate responses to social or emotional stimuli (Jankord and Herman, 2008, Herman et al., 2005). The role of each brain region in stress responses will be further examined in Chapter 4. Ultimately, it is the combination of excitatory and inhibitory signalling between different brain regions, which can be further modulated by other signalling molecules (e.g. other neurotransmitters, neuropeptides and neurosteroids), that mediate HPA axis activation and its subsequent termination (Fig 1.2).

Activation of the HPA axis:

Activation of the HPA axis is rapid and generally occurs directly via neural circuits. Physical and psychological stressors are processed by different neural circuitries, but may overlap in some instances (Godoy et al., 2018). In general, physical stressors such as inflammation or pain signal through direct noradrenergic and adrenergic projections originating from the nucleus of the solitary tract (NTS) of the brainstem to the PVN (Ulrich-Lai and Herman, 2009). CRH-producing mpPVN neurones express α1-adrenergic receptors and are directly activated by this noradrenaline release (Cole and Sawchenko, 2002, Itoi et al., 1994). Psychogenic stressors activate more complex pathways as they require the appraisal of the stressor, therefore rely heavily on the activation of several limbic structures and the integration of signals from these limbic structures (reviewed in (Godoy et al., 2018, de Kloet et al., 2019)). For instance, activation of sequential GABA synapses in the

different subregions of the amygdala, followed by the integration of these signals alongside those from other brain regions (such as the hippocampus and the PFC) at the peri-PVN region, eventually results in the production of CRH in the mpPVN neurones (Roland and Sawchenko, 1993, Ulrich-Lai and Herman, 2009, Bains et al., 2015). Depending on the stressor, other neural mechanisms, such as direct serotonergic inputs from the raphe nuclei to the PVN (Herman et al., 2003, Sawchenko et al., 1983), and non-neural mechanisms such as the action of cytokines and inflammatory factors (e.g. nitric oxide) on the PVN may also play a role in initiating stress responses (summarised in (Rivest, 2001)).

Inhibition of the HPA axis:

Inhibition of the HPA axis occurs over a longer time scale, and is controlled by both neuronal signalling mechanisms, as well as other slower mechanisms that involve gene expression changes (Ulrich-Lai and Herman, 2009). One of the key mechanisms is the negative feedback inhibition mediated by glucocorticoids binding of GR in the PVN, resulting in the rapid release of endocannabinoids, which consequently inhibits glutamate release and thereby reducing excitatory drive to CRH-producing mpPVN neurones (Di et al., 2003). Apart from this direct inhibition on the PVN, indirect inhibition via glucocorticoid signalling can originate from other limbic structures such as the hippocampus, where signals are then subsequently transmitted to the peri-PVN region. GABA neurotransmission is also an important aspect of HPA axis inhibition, as the PVN is directly innervated by GABA projections from various brain regions, including the bed nucleus of the stria terminalis (BNST; which is a centre for integration of limbic signals) (Cullinan et al., 1993, Herman et al., 2016). GABA neurotransmission can further be modulated by the rapid action of neuroactive steroids, which will also be further elaborated in sections 1.3.2 and 1.3.7.

Although the characterisation of above-mentioned stress activation and inhibition pathways have been carried out in rodents, stress response pathways are generally well-conserved across species and therefore show good translatabilty to humans (Cryan and Holmes, 2005, Hariri and Holmes, 2015). For instance, the neural circuits that regulate learning and memory in response to fear, a form of stressor, show striking parallels between the human and rats and involves the amygdala (reviewed in (Hariri and Holmes, 2015)). Nonetheless, apart from mechanisms of HPA axis activation and inhibition mentioned here, which have generally been

determined using controlled experiments using single stressors, there also exists a plethora of mechanisms that can further modulate glucocorticoid action and HPA axis functioning, such as sex, circadian rhythm, and age. Most importantly, the processes summarised here also represent the classic stress response to a single bout of acute stress, and the same processes may not occur in response to chronic or severe stressors, which can have a cumulative effect (Herman et al., 2016). These factors add further layers of complexity to the patterns of stress response, and this becomes especially salient in higher organisms with more complex social lives such as humans. In humans, apart from the more complex nature of stressors, greater cognitive abilities also allow for the capacity to process stressful situations in ways that are different as compared to that of rodents, and the activation of the stress response may lead to different outcomes in terms of vulnerability and resilience to stress-related pathology.

1.2.2 Allostasis and stress-related pathology

As mentioned earlier, the SNS and HPA axis can have very different patterns of response, varying in magnitude based on the type, frequency, and duration of the stressor, and also during different periods throughout an organism's life course. Chronic stress, for instance, requires the body to employ new or modified coping strategies that can be different from that for acute stress (Herman, 2013). It is known that with repeated stress, habituation can occur, where the magnitude of HPA axis activation to a stressor declines with repeated exposure to that same stressor (Grissom and Bhatnagar, 2009, Natelson et al., 1988). However, different degrees of habituation can occur for different types of stressors, and in certain cases, habituation may completely fail to happen (Figueiredo et al., 2003a), or sensitisation of stress responses occur instead. The HPA axis then becomes hyper-responsive, which is associated with the development of pathologies (Belda et al., 2015, Natelson et al., 1988). Herein lies two complexities inherent to stress responses – firstly, the dynamic nature of the stress response, in that it changes according to different contexts, secondly, the problem of pathological stress responses – why is it that some stress responses are adaptive, and some are pathological, and at what point does the stress response start to become pathological?

These two complexities in stress responses can be addressed using a framework known as "allostasis", which literally means "maintaining stability through change". Activation of the SNS and HPA axis represent part of the process of "allostasis", as

they both constitute an alteration of physiology to restabilise the body in response of an acute challenge. Unlike the traditional concept of “homeostasis”, which focusses on the return of physiological systems to an invariant set point, “allostasis” emphasises dynamic and flexible regulation, where the set-point is adjustable according to different contexts (Ramsay and Woods, 2014).

There are two key aspects of the allostasis framework (Fig 1.2). Firstly, apart from glucocorticoids and catecholamines produced from the HPA axis and SNS, respectively, the process of allostasis is controlled by a complex network of other inter-dependent mediators, including cytokines, neurotransmitters, neuropeptides, and neuroactive steroids etc. These mediators can all interact in a highly inter-dependent manner, such that a change in one mediator may lead to subsequent changes in all the other mediators. This implies that stress responses are inherently complicated, and various different outcomes can transpire depending on the context. Secondly, the process of allostasis is one that comes with a cost. Insofar as glucocorticoids are helpful to deal with stressors, if their catabolic functions are allowed to persist indefinitely, an enormous burden is placed on the body. This is known as the “allostatic load”, and the body is said to be in an “allostatic state”. Given that “allostatic load” is progressive and cumulative, at this point, symptomatic disease might not yet show and these deviations are reversible if caught in time (McEwen, 2004). However, it is a fragile state and if allowed to persist, a state of “allostatic overload” ensues which can lead to disease (McEwen, 2004)(Fig 1.2).

In the context of the HPA axis, an allostatic state is characterised as a constant state of HPA hyperactivity or hyporesponsiveness. Optimal glucocorticoid action has been described as an inverted U-shaped curve, where both hyper- and hypo-secretion can be problematic (De Kloet et al., 1998, Diamond et al., 1992). For instance, in the brain, excessive glucocorticoids can decrease hippocampal neurogenesis and increase hippocampal apoptosis (Gould and Tanapat, 1999, Kino, 2015). Hippocampal neuronal morphology can also be also affected, characterised by decreases in dendritic length and dendritic branch points (Tata and Anderson, 2010). These can not only lead to deficits in learning and memory processes, but can also further worsen the dysregulation of the HPA axis as hippocampal neurogenesis is critical for hippocampal-mediated glucocorticoid negative feedback (Snyder et al., 2011). On the other hand, hypoactivation of the HPA axis and inappropriately low production of glucocorticoids to a stressor are harmful as well, as

they increase susceptibility to inflammatory conditions, where the body is ill-prepared to face challenges, thereby increasing vulnerability to pathology (Diamond et al., 1992; Reber et al., 2007)

In humans, HPA axis dysregulation can be determined by tests such as the dexamethasone suppression test or the dexamethasone/CRH test (Heuser et al., 1994). Both hyper-responsiveness and hyporesponsiveness are known to occur following states of chronic stress (Heim et al., 2000, Fries et al., 2005). In clinical studies, HPA axis dysregulation is strongly associated with various disorders. Hyper-secretion of cortisol is strongly associated with metabolic disease, such as visceral obesity, type 2 diabetes and hypertension (Tirabassi et al., 2014, Min, 2016), as well as mood disorders such as major depression or bipolar disorder (Plotsky et al., 1998), whilst hypoactivation of the axis is more commonly observed for autoimmune diseases (Tsigos and Chrousos, 2002) and post-traumatic stress disorder (PTSD) for instance (Daskalakis et al., 2016).

Among potential mechanisms of such allostatic states of HPA axis dysregulation are (1) altered biosynthesis or metabolism of the key mediators of the HPA axis (e.g. CRH, ACTH, glucocorticoids), (2) altered availability and action of these mediators due to alteration of local concentrations (e.g. by local glucocorticoid metabolism), (3) altered receptor sensitivity in the key regions associated with the HPA axis (e.g. CRH receptors in the anterior pituitary, ACTH receptors in the adrenal glands), (4) altered feedback sensitivity of the HPA axis (which may stem from altered GR sensitivity in areas associated with feedback inhibition e.g. hippocampus, hypothalamus or anterior pituitary or any other mechanisms mentioned in the previous section) (Heim et al., 2000), and (5) other morphological, neural circuitry, or neurotransmitter changes in various brain regions which can affect the activation of the HPA axis (e.g. structures of the limbic system amygdala or hippocampus cortex). Throughout this thesis, these common underlying causes will be revisited when discussing stress-related pathology.

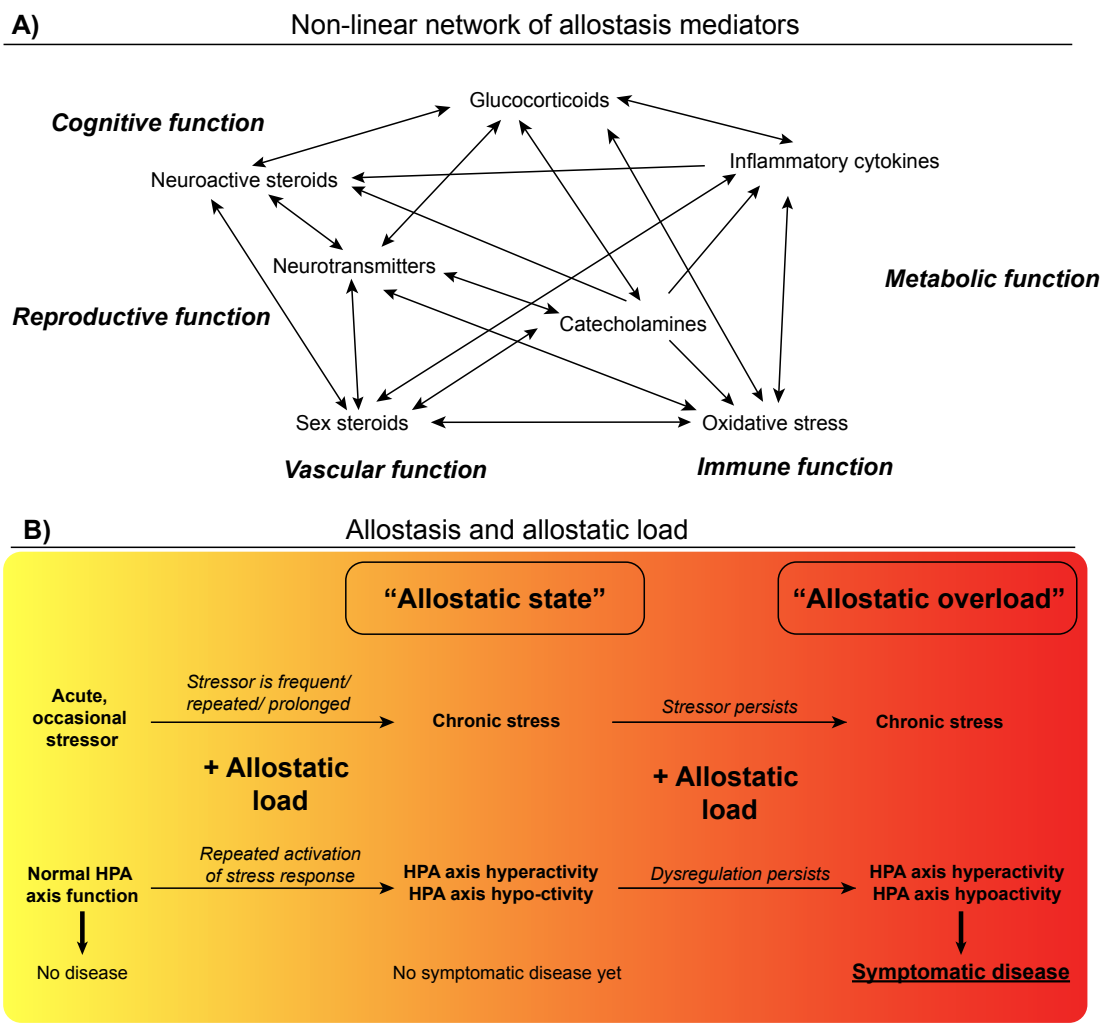


Figure 1.2: Allostasis mediators and allostatic load. (A) A key aspect of allostasis is the presence of a non-linear network of allostasis mediators, where one a change in one mediator can result in a change in output of another mediator in the system (McEwen, 2006). Together, they influence the function of the cognitive, reproductive, metabolic, vascular, and immune systems etc. Diagram adapted from Fig 2 of McEwen, 2006. (B) Allostatic load accumulates following stress to result in an "allostatic state". Whilst an "allostatic state" may not necessarily result symptomatic disease, it is a fragile state and if allowed to persist, will result in "allostatic overload" where disease occurs. HPA axis dysregulation begin to occur in an "allostatic state" and may worsen in "allostatic overload".

1.3 Steroids and the stress response

This thesis is focussed on investigating the role of steroids in modulating the outcomes of stress, especially in the context of gestational stress. Steroids are a class of lipophilic signalling molecules that are all derived from a common cholesterol precursor, and constitute key components of the neuroendocrine response to stress (Charmandari et al., 2005). They include the sex hormones (testosterone, progesterone, and oestradiol), glucocorticoids, mineralocorticoids and their downstream metabolites (Fig 1.3).

The first step of steroid synthesis is the transport of cholesterol from the cytoplasm into the mitochondria, which is controlled by the enzyme, steroidogenic acute regulatory protein (StAR, *Star*) (Fig 1.3). Once cholesterol is inside the mitochondria, p450 side-chain cleavage enzyme (p450scc; *Cyp11a1*) converts it to pregnenolone, which is considered the master precursor steroid. Pregnenolone is subsequently converted to downstream steroids such as progesterone, deoxycorticosterone (DOC), and testosterone. Steroids can be interconverted by steroidogenic enzymes such as 3α- and 3β-hydroxysteroid dehydrogenase (3α-HSD; *Akr1c9* and 3β-HSD; *Hsd3b*) and 5α-reductase (both *Srd5a1* and *Srd5a2* isoforms) to various metabolites that may seem structurally similar, but in fact possess very different functional properties. Each individual steroid metabolite is a distinct component of the non-linear network of mediators of allostasis mentioned in section 1.2.2.

This section will focus on the molecular characteristics of steroids and how they exert their effects on a cellular and system level. On a cellular level, steroid action is influenced by two factors, downstream interaction with receptors (section 1.3.1), as well as the intricate control of their local concentrations in target tissues or cells (section 1.3.2) (summarised in Fig 1.4). On a system level, steroid action can be influenced by various physiological factors, such as circadian rhythms (section 1.3.3), sex (section 1.3.4) or developmental stages (section 1.3.5). Lastly, steroids that specifically target neuronal signalling (i.e. neuroactive steroids) and their role in the stress response will be briefly introduced (section 1.3.6). A common theme emerge from these molecular processes, in that they act in concert to ensure that responses to stress occur in a controlled and regulated manner (Herman, 2013).

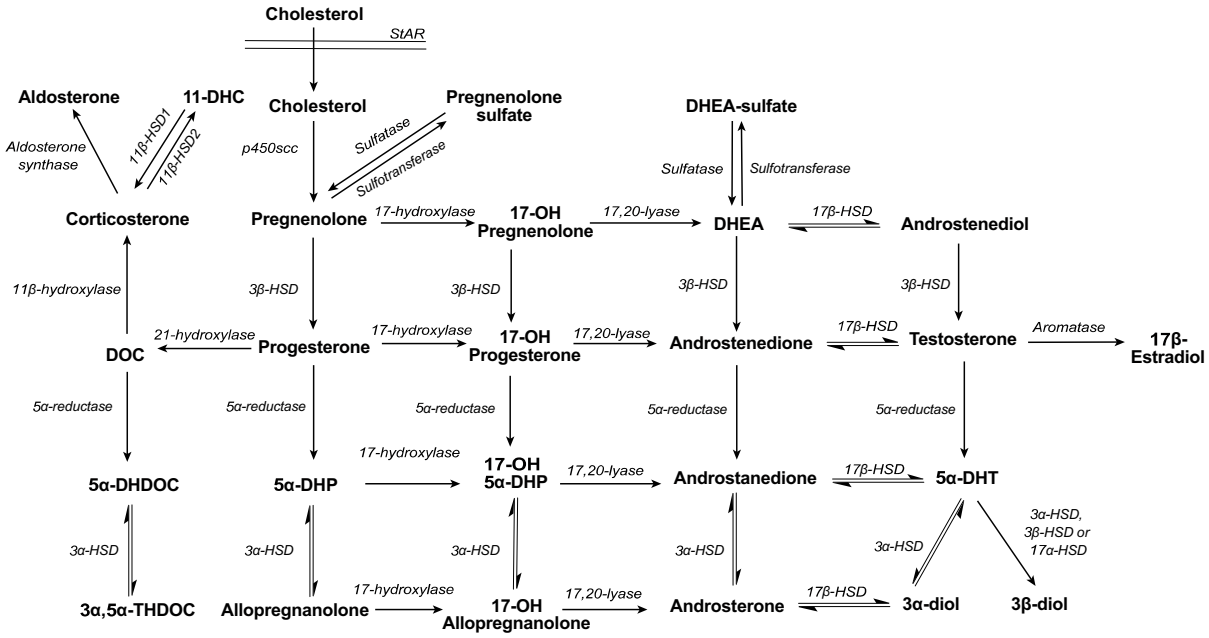


Figure 1.3: Steroid interconversion diagram. Cholesterol enters the mitochondria (facilitated by the steroidogenic acute regulatory protein; StAR) and is cleaved by the p450 side-chain cleavage enzyme (p450scc) into pregnenolone. Pregnenolone can be converted to progesterone by 3β-hydroxysteroid hydrogenase (3β-HSD), which can subsequently be converted to deoxycorticosterone (DOC), then corticosterone. Corticosterone can be converted to the mineralocorticoid aldosterone by aldosterone synthase. Progesterone and DOC can also undergo two sequential A-ring reduction steps to form the 5α,3α-reduced steroids, allopregnanolone and tetrahydrodeoxycorticosterone (THDOC), respectively. These reduction steps are catalysed by the enzymes 5α-reductase (both isoforms of 5α-reductase 1 and 2) and 3α-hydroxysteroid dehydrogenase (3α-HSD), respectively. Pregnenolone and progesterone can be converted to androgens by a two-step pathway, catalysed by the 17-hydroxylase and 17,20-lyase activities of a single enzyme, cytochrome P450c17. Testosterone can similarly be reduced to form dihydrotestosterone (DHT), and subsequently 3α-androstanediol (3α-diol) and 3β-androstanediol (3β-diol). DHT can also be produced in a “backdoor pathway” without the need for testosterone production, through a pathway mediated by 17-OH progesterone, 17-OH DHP, 17-OH allopregnanolone, androsterone and 3α-diol, catalysed by the same common enzymes (reviewed in (Auchus, 2004). Lastly, testosterone can be converted to 17β-oestradiol via the actions of aromatase (*Cyp19a1*). Chemical modifications such as sulfation may also change the properties and functions of steroids such as dehydroepiandrosterone (DHEA) and pregnenolone. Given these steroidogenic enzymes catalyse several reactions and may be bi-drectional, steroid production is therefore determined by a multitude of factors such as the availiability of the precursor, concentrations of the enzymes and co-factors (reviewed in (Miller and Auchus, 2011))

1.3.1 Molecular mechanism of steroid actions

At a molecular level, steroids can act via both genomic and non-genomic pathways, in both the periphery and in the brain. For genomic actions, steroids exert their physiological effects via classical steroid receptor actions and involves alteration of gene expression. Non-genomic actions of steroids are mediated through receptor-dependent or independent pathways, usually over a much shorter timescale compared to genomic actions.

1.3.1.1 Genomic action of steroids

Steroid receptors such as GR, MR, progesterone receptors (PR: *Pgr*), androgen receptors (AR; *Ar*), and oestrogen receptors (both ERα; *Esr1* and ERβ; *Esr2* isoforms) are nuclear receptors which act as ligand-dependent transcription factors. In the absence of ligand, they exist in the cytoplasm as part of an inactive multi-protein complex, bound to protein chaperones such as heat shock proteins (HSPs) (section 1.3.1.3). The binding of the steroid ligand results in a conformational change and the dissociation of receptors from the HSPs. The activated steroid-receptor complex then dimerises and translocates to the nucleus, interacting with steroid responsive elements to enhance or repress the transcription of genes (Beato, 1989) (Fig 1.4).

1.3.1.2 Glucocorticoid actions via MR and GR

Glucocorticoid action, specifically, is mediated by the steroid receptors, MR and GR. Although glucocorticoids bind to both MR and GR, the outcomes of binding to the two receptors are different, due to differences in binding affinities, regional expression patterns, as well as the target genes they regulate (Datson et al., 2001). As MRs have a much higher affinity for glucocorticoids than GRs, under basal conditions, MRs are preferentially occupied (Reul and de Kloet, 1985). This continuous MR activation at a basal state allows for the regulation of electrolyte balance, and the control of blood pressure and sympathetic drive (Gomez-Sanchez and Gomez-Sanchez, 2012). In contrast, GRs are usually only occupied when glucocorticoid levels are higher, for instance following stress exposure, or when there is a circadian rise in glucocorticoids (de Kloet et al., 2005, Lightman and Conway-Campbell, 2010, Reul and de Kloet, 1985). GR therefore plays a more prominent role in inhibition of the HPA axis via negative feedback, and can act for

instance, by the transcriptional downregulation of CRH and AVP upon glucocorticoid binding (Keller-Wood and Dallman, 1984, Tasker et al., 2005).

Additionally, there are differences in the brain expression pattern of MR and GR. GRs are present in most brain regions, while MRs have a much more limited distribution and are more concentrated in the limbic regions, especially the hippocampus (Reul and de Kloet, 1986). Thus, a balance between MR- and GR-mediated actions in the limbic system neurones must be maintained for the HPA axis to function properly (De Kloet et al., 1998, de Kloet, 2014). Imbalance could be one of the underlying molecular mechanisms of HPA axis dysregulation, increasing the vulnerability to affective disorders (Harris et al., 2013).

1.3.1.3 Role of chaperone proteins

The specificity of the genomic action of steroids is also contingent on the action of protein chaperones in the protein complex (Fig 1.4). The genomic action of GR for instance, is regulated by heat shock proteins (HSPs) such as HSP90 and HSP70, and also the FK506-binding proteins (FKBP), such as FKBP51 (*Fkbp5*) and FKBP52 (*Fkbp4*). HSPs have a role to play in GR intracellular trafficking and activation, while FKBPs regulate receptor-ligand binding and also nuclear translocation (reviewed in (Pratt et al., 2004, Sivils et al., 2011)). FKBP51 is bound to the inactivated protein receptor complex. When glucocorticoid binds to the receptor, the conformational change causes FKBP51 to dissociate and FKBP52 to bind instead, which then allows for its nuclear localisation (Galigniana et al., 2001). The actions of FKBP51 and FKBP52 are thus antagonistic, with FKBP51 generally acting as a negative modulator of steroid receptor activity, inhibiting nuclear translocation; while FKBP52 on the other hand, positively modulates nuclear translocation (Wochnik et al., 2005). Notably, FKBP51 plays a prominent role in the regulation of stress responses, as FKBP51 expression is found to increase in the amygdala and PVN of adult mice following acute and prolonged stress (Scharf et al., 2011), whilst FKBP51 knockout mice show decreased stress responses and lower basal corticosterone concentrations (Hartmann et al., 2012, Hoeijmakers et al., 2014). It is also worth noting that FKBP51 and FKBP52 are not only involved in GR signalling, but are also required for the optimal function of MR, PR and AR (reviewed in (Sivils et al., 2011, Stechschulte and Sanchez, 2011)).

1.3.1.4 Non-genomic action of steroids

It has become clearer that steroids do not only act by classic steroid receptor action, as there are many steroid-mediated cellular responses that occur within minutes, which is generally considered too short a time frame for genomic effects to occur (Simoncini and Genazzani, 2003, Makara and Haller, 2001). These downstream effects that are rapid and do not affect gene expression directly, are defined as non-genomic effects of steroids (Lösel and Wehling, 2003). Having both genomic and non-genomic signalling pathways allows for diversity in the way in which steroids may impact the HPA axis. There are three major non-genomic mechanisms where steroids can exert effects, these are through 1) cytosolic steroid receptors, 2) membrane-bound steroid receptors, or 3) non steroid-receptor mediated pathways.

Cytosolic steroid receptors: The non-genomic effect of steroid action can occur concomitantly with the classic genomic effects, where the conformational changes following ligand binding to cytosolic receptors can also lead to the release of kinases, which can activate signalling pathways. For instance, the binding of glucocorticoids to GR releases the tyrosine kinase, Src which was originally in the protein chaperone complex, allowing it to be free to phosphorylate its downstream targets (Solito et al., 2003). Glucocorticoids also exert their anti-inflammatory properties through a non-genomic pathway, as glucocorticoid binding to GR disrupts the formation of a T-cell receptor complex, leading to decreased immune function (Löwenberg et al., 2007).

Membrane-bound steroid receptors: Most of the rapid changes in neuronal excitability and activity following glucocorticoid binding are mediated by membrane-bound MR and GR receptors (Groeneweg et al., 2011). In addition to the slower transcriptional downregulation of CRH and AVP expression in the PVN via nuclear GR, HPA axis negative feedback inhibition is also heavily dependent on the action of glucocorticoids on membrane GR. Using male Sprague Dawley rats, it has been found that glucocorticoid- membrane GR binding induces endocannabinoid synthesis, which acts in a retrograde manner to suppress the excitatory drive to the CRH-producing cells in the PVN (Di et al., 2003, Evanson et al., 2010). This is also the case for the membrane-bound PR, where progesterone binding can lead to the activation of several non-nuclear signalling pathways such as the extracellular signal-related kinase (ERK) pathways, cAMP/protein kinase A (PKA) signalling, Ca^{2+}

influx/PKC activation, or the phosphatidylinositol 3-kinases/protein kinase B (commonly known as PI3K/Akt) pathway, among many others (Singh et al., 2013).

Non steroid receptor-mediated: Steroid hormones can also directly exert effects on cell membranes by altering membrane permeability and fluidity (Whiting et al., 2000). In immune cells for instance, glucocorticoids at high concentration can be intercalated into plasma and mitochondrial membranes, activating various membrane-associated proteins or calcium signalling (Buttgereit and Scheffold, 2002). Alternatively, neuroactive steroids may have rapid effects by acting allosterically on receptors that are not classic steroid receptors, such as transmembrane ion-channel proteins (e.g. NMDA or $GABA_A$ receptors), thereby modulating the effects of the neurotransmitters glutamate and GABA on brain function (section 1.3.6) (summarised in (Groeneweg et al., 2011, Gunn et al., 2011)).

1.3.2 Regulation of local steroid availability

The actions of glucocorticoids and steroids are eventually dependent on local concentrations in target tissues and cells. Local regulation can occur via a few different mechanisms, and can occur quickly in response to perturbations such as stress. Firstly, the concentration of steroids in local organs can be dependent on binding to carrier proteins, as it limits the amount of steroids that can freely diffuse into cells to act on intracellular receptors (section 1.3.2.1) (Hammond, 2016). Secondly, apart from steroids produced by the classic endocrine organs (e.g. adrenal glands and gonads), it is also known that there exists enzymatic machinery in various organs that can inactivate or activate steroids (e.g. 11β-hydroxysteroid dehydrogenase enzymes; section 1.3.2.2), or produce downstream steroids from circulating, adrenal-derived steroid precursors (e.g. 5α-reductase or 3α-HSD; section 1.3.2.3). This *in situ* production of steroids in the target organs allow for steroids to act in an intracrine (i.e. steroid synthesis and signalling are occurring in the same cell) or a paracrine (i.e. steroid synthesis for action in a neighbouring cell) manner, without requiring secretion into the circulation. The implication of this local regulation or production is that changes observed in circulating levels of steroids may not necessarily equate to changes in local levels in the brain.

1.3.2.1 Binding or carrier proteins

As steroids are lipophilic, they are transported in the bloodstream bound to proteins such as albumin, sex hormone-binding globulin (SHBG; *Shbg*) and corticosteroid-binding globulin (CBG; *Serpina6*) (Hammond, 2016). The binding of steroids to these proteins therefore affect their availability to act on their cognate receptors, since only free unbound steroids may freely diffuse through the plasma membranes to exert cellular actions (Mendel, 1989).

The levels of SHBG (which binds to androgens and oestrogens) and CBG (which binds to glucocorticoids and progesterone) can change according to different conditions, such as during stress or inflammation, allowing for more dynamic control of the access of steroids into target tissues and cells. In humans, CBG levels are altered in a sex-dependent manner following the Trier Stress Test (Kumsta et al., 2007), while in mice, deficiencies in CBG also affect corticosterone responses to stress and hippocampal-dependent memory processes (Minni et al., 2012). Serum albumins bind to most steroids without much specificity and with low affinity, however, they exist in high concentrations in blood, thus can additionally modulate tissue steroid concentrations as they can buffer drastic changes when plasma steroid concentrations increase transiently (Hammond, 2016).

Although it is often assumed that steroids that are bound to these proteins are inactive, there may in fact be additional ways in which they may act, as bound steroids can activate membrane steroid receptors directly, or can still enter the cell by facilitated endocytosis (Chen and Farese, 1999). Additionally, although unbound steroids freely diffuse, there is also evidence of membrane transporters that are present, especially at the blood brain barrier that can control the influx and efflux of steroids such as cortisol, further complicating the modulation of intracellular glucocorticoid concentrations in brain regions (Mason et al., 2008).

1.3.2.2 11β-hydroxysteroid dehydrogenase (HSD) enzymes

The enzymes 11β-HSD1 (*Hsd11b1*) and 11β-HSD2 (*Hsd11b2*) control local pre-receptor metabolism of glucocorticoids, converting glucocorticoids to their inactive 11-keto form and vice versa. 11β-HSD1 functions primarily to convert inactive 11-dehydrocorticosterone to corticosterone (or cortisone to cortisol in humans), while

11β-HSD2 catalyses the reverse reaction and inactivates corticosterone to 11-DHC (Chapman et al., 2013, Agarwal et al., 1989, Brown et al., 1993) (Fig 1.4).

In the brain, 11β-HSD1 is highly expressed in regions of the brain that also express GR, including the PVN and the hippocampus, and hence it has been suggested to play a role in regulating negative feedback control of the HPA axis, as well as regulating glucocorticoid effects on mood, learning, and memory (Holmes and Seckl, 2006). In contrast, the expression of 11β-HSD2 has been predominantly associated with peripheral tissues such as the kidney and the placenta. In the kidney, 11β-HSD2 prevents overt binding of corticosterone to MR, and enabling aldosterone to bind to the receptor to exert its action on salt and water balance (Edwards et al., 1996). In studies of the adult rat brain, 11β-HSD2 is found to be expressed in a few, discrete nuclei also involved in the regulation of salt/water balance and blood pressure (Robson et al., 1998).

During pregnancy, 11β-HSD2 is also highly expressed in the placenta, where it acts as a protective barrier to inactivate excess maternal glucocorticoids, hence preventing excessive glucocorticoids from reaching the foetus. 11β-HSD2 is also expressed in the developing foetal brain, possibly acting as a further layer of defense against excessively high levels of corticosterone (reviewed in (Holmes and Seckl, 2006)). These will be further elaborated in section 1.4.

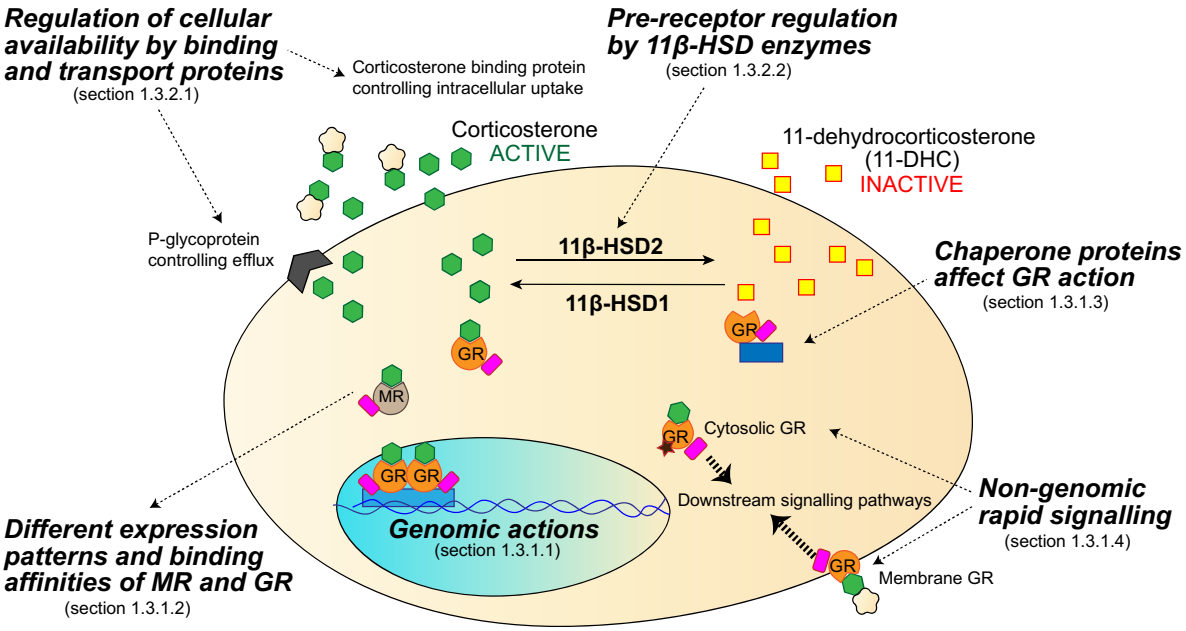


Figure 1.4: Summary of some of the factors affecting the molecular actions of steroids, using the example of glucocorticoids. Glucocorticoid action can be affected by its different binding affinities to MR and GR, which differ in downstream targets. The nature of the receptor (e.g. whether it is cytosolic or located in the membrane), and its association with chaperone proteins, can also affect whether the downstream action of steroid ligand-receptor binding is genomic or non-genomic. There are also mechanisms to control their local concentrations in cells, by carrier proteins such as CBG, transporter proteins such as p-glycoprotein and pre-receptor regulation by the action of 11β-HSD enzymes. In rats, 11β-HSD2 converts active corticosterone to inactive 11-DHC (which is unable to bind to MR or GR), while 11β-HSD1 mainly reactivates 11-DHC.

1.3.2.3 Neurosteroidogenesis in the brain

The brain has the enzymatic capability to synthesise steroids *de novo*, evident from the observations in rats that steroid levels can change rapidly independent of peripheral concentrations, and can still be detected even upon removal of the adrenal glands or gonads (Compagnone and Mellon, 2000, Corpechot et al., 1981, Corpechot et al., 1983). These steroids that are produced and have local actions in the brain are termed as “neurosteroids” (Baulieu, 1991).

Neurosteroids are produced in the neurones and glia, and have a wide range of influences on the brain, ranging from regulating neuronal survival, differentiation, myelination, dendritic growth, and synaptogenesis during development, to the basal regulation of circadian rhythms, mood, memory, and sleep, or the activation and inhibition of the stress response (Mellon, 2007, Borowicz et al., 2011). They are present in higher concentrations in tissue from the nervous system than in the plasma, and their levels are known to further increase rapidly following conditions such as injury and stress (Purdy et al., 1991, Paul and Purdy, 1992, Barbaccia et al., 1998).

These steroids work in an paracrine, as well as an autocrine manner to regulate stress responses (Girdler and Klatzkin, 2007). At the same time, there also exists feedback loops which regulate their own synthesis (Meethal et al., 2009). There has also been thorough characterisations of the neurosterodogenic enzyme machinery in the brain of rats and mice, and expression of these enzymes has been found to be region-, neurone subtype-, and developmental timepoint- specific (Compagnone and Mellon, 2000, Agis-Balboa et al., 2006, Mellon and Griffin, 2002). The molecular action of neurosteroids is elaborated in section 1.3.7.

1.3.3 The influence of circadian rhythms

All organisms have intrinsic biological “clocks” that regulate biochemical, physiological, and behavioural changes throughout the day, to control essential processes such as the sleep-wake cycle and to meet the body’s energetic demands. In neuroendocrine systems, hormonal production shows both circadian patterns (i.e. a cycle that occurs once a day) and ultradian patterns (i.e. cycles that repeat throughout a 24 hour period).

In terms of the HPA axis, there is a strong circadian rhythm where plasma concentrations peak just prior to the active part of the day, which in diurnal animals (e.g. humans) is in the early morning, and for nocturnal animals (e.g. rodents), in the early evening (Gnocchi and Bruscalupi, 2017, Watts et al., 2004). This is controlled by the central clock within the suprachiasmatic nuclei (SCN) of the hypothalamus (Nader et al., 2010, Ralph et al., 1990). In addition to and underlying this circadian rhythm, there also exists an ultradian rhythm, where there are oscillatory pulses of ACTH and glucocorticoid throughout the day (Veldhuis et al., 1989). In the rat, this exists as an approximately hourly rhythm of corticosterone secretion that is regulated by alternating activation and inhibition of the HPA axis (Lightman et al., 2008). This is controlled by various negative feedback mechanisms, which include dynamic pituitary-adrenal interactions, or even chaperone proteins (Walker et al., 2010, Gjerstad et al., 2018). Disruption of these of glucocorticoid circadian rhythms therefore can be a manner in which aberrant stress responses and disease can develop both in humans and in rodents, reviewed in (Spiga and Lightman, 2015).

Circadian cycles have also been described for sex steroids such as testosterone (Alvarez et al., 2008) and progesterone (Nakao et al., 2007, Bailey, 1987) in mouse studies. In terms of experimental work, the presence of these rhythms mean that the time of the day should always be considered an important factor when designing/conducting experiments and reporting data (Gumz, 2016).

1.3.4 The influence of sex

Apart from expected differences in the concentration of sex steroids, where males have greater concentrations of testosterone and females have higher concentrations of progesterone and oestradiol, sex differences are present in neuroendocrine responses to stress. In rodent studies, females are generally known to have greater stress responses in comparison to males (Sze et al., 2018, Bale and Epperson, 2015, Handa et al., 1994, Tinnikov, 1999). In humans, such patterns are less consistent as measurements of HPA axis and SNS output (e.g. plasma cortisol, heart rate, blood pressure) show far more variability, as observed in a systematic review by (van der Voorn et al., 2017). However, men and women still display different risk profiles for stress-related diseases. For instance, women are more prone to developing anxiety, depression, panic and eating disorders, while men show more antisocial behaviour and substance abuse (Altemus et al., 2014).

Women also report higher incidences of stress, which may be attributed to underlying differences in stress responsivity (Wellman et al., 2018).

These sex differences can be explained by the organisational-activational theory of sexual differentiation, and are driven by differences in sex steroid concentrations between males and females, as reviewed in (McCarthy et al., 2012, Arnold, 2009). Sex steroid concentrations in males and females are inherently different due to the presence of ovaries in females and testes in males, which in males are in turn are determined by the SRY gene on the Y chromosome. During a critical developmental period in foetal life, steroids organise the brain by the regulation of gene expression, epigenetics and protein synthesis etc., which result in permanent changes in neuroanatomy and circuitry (Bingham and Viau, 2008). These effects are known as the “organisational” effects of sex hormones. In rats and mice, a testosterone surge occurs around gestational day (GD) 18 where its levels are significantly higher than levels in female siblings (Ward et al., 2003). There is another additional surge a few hours after birth, but levels then remain low postnatally until puberty (Weisz and Ward, 1980). This testosterone surge prenatally and at birth is recognised to be the dominant organising signal in rodents (Lenz et al., 2012). In humans, a prenatal testosterone surge also occurs prenatally at about weeks 8 to 24 of gestation, however, in contrast to rats and mice, another testosterone surge also occurs in the first few months after birth before decreasing at about 6 months until puberty, where levels rise again ((Winter et al., 1976) and reviewed in (Bell, 2018)). Therefore, “organisational” effects can also be considered to occur during puberty, where there are further maturational changes in the neural circuitry of the brain, due to the epigenetic effects of gonadal hormones (reviewed in (Morrison et al., 2014)).

Apart from this “organisational” effect during these critical windows which alter neuroanatomy and circuitry, during adulthood, there are direct “activational” effects of these steroids on cellular function. “Activational” effects are only present when the steroids are present, and are especially pronounced after puberty, and are considered as mechanisms to maintain sex differences in adulthood. Sex steroids are part of the hypothalamic-pituitary-gonadal axis (HPG), which controls reproduction and fertility, but also has interactions with the HPA axis and is able to modulate stress signalling (Oyola and Handa, 2017, Acevedo-Rodriguez et al., 2018). In general, testosterone suppresses stress reactivity while oestradiol seems to enhance HPA axis responses (Viau and Meaney, 1996). High oestradiol

concentrations result in elevated basal corticosterone, enhanced and prolonged corticosterone secretion following stress, seemingly via an increased adrenal sensitivity to ACTH, which may explain why stress responsivity and plasma corticosterone concentrations are at maximum levels at pro-estrus in female rodents (Atkinson and Waddell, 1997, Viau and Meaney, 1991). Progesterone also play a modulatory role in regulation of the female HPA axis, but is likely to be inhibitory instead, as it prevents the enhancing effects of oestrogen on ACTH release during stress in rats (Viau and Meaney, 1991, Roy et al., 1999). A further complexity exists in that testosterone can also exert its actions via its conversion to oestradiol, catalysed by the enzyme aromatase, which show different activities in males and females, which may lead to differences in various neurobehavioural outcomes (Shay et al., 2018). Sex differences observed in male and female HPA axis responses in adulthood are therefore a result of permanent “organisational” effects and more transient “activational” effects of sex steroids.

The implications of these sex differences when investigating the role of steroids in stress axis regulation is that males and females should be analysed separately as there is likely to be mechanistic differences between them, whether or not there are phenotypical differences. This is in line with the National Institute of Health directive which calls for the need to include both sexes in animal experiments (Clayton and Collins, 2014). Moreover, as sex differences in neuroanatomy and circuitry emerge at a young age, it is necessary to consider sex even during foetal stages.

1.3.5 The influence of developmental stage

Neuroendocrine responses to stress are not homogenous across different ages, different outcomes can emerge when individuals are exposed to stress at different stages of their lives (Lupien et al., 2009, Koenig et al., 2011, Bale and Epperson, 2015). Across different stages of rodent development, the basal concentrations of corticosterone and sex steroids in the brain and circulation are also not static (Taves et al., 2015, Konkle and McCarthy, 2011).

Firstly, the foetal HPA axis in rats develops at about GD16 (Lugo and Pintar, 1996, Brunton, 2013), and are found to be responsive to stressors as early as GD18 (Ohkawa et al., 1991b). A similar increased HPA activity is observed in the third trimester in human pregnancies, where production of glucocorticoids and neuroactive steroids peak near parturition (Wood and Keller-Wood, 2016, Morsi et

al., 2018). The foetal HPA axis does not participate in the stereotypical “stress response” *per se*, but instead mediates processes such as the timing of birth, and prepares the foetus for birth and the extrauterine life (Wood and Keller-Wood, 2016) (elaborated in section 1.5).

After birth, there exists a stress hyporesponsive period (mostly during the first two weeks in life for rats), as excess glucocorticoids can disrupt the normal development of glucocorticoid-sensitive brain regions (Sapolsky and Meaney, 1986). Neonatal rats have low basal levels of corticosterone, and fail to show an adult-like increase in plasma corticosterone or ACTH concentrations upon exposure to a variety of stressors (including ether, handling, heat, electric shock, and cold stressors), (Dallman, 2000, Sapolsky and Meaney, 1986). Studies from rats (Walker et al., 1990) and mice (Schmidt, 2010) both suggest that it is likely that the quiescent HPA axis during this stage is due to suppression of HPA activity at the level of the anterior pituitary, as well as decreased sensitivity of the adrenals to ACTH. In the brain, whilst GR expression in the hippocampus is fairly low at birth, it increases significantly over the first two weeks of life, with the highest levels of expression peaking at PND 12, which potentiates negative feedback inhibition of the HPA axis (Schmidt et al., 2003).

The HPA axis starts to mature during puberty, in tandem with the maturation of brain circuitry in the limbic regions, reviewed in several rodent and human studies in (Eiland and Romeo, 2013). In contrast to the stress hyporesponsive period during neonatal life (in rats) and childhood (in humans), the peri-pubertal period is often characterised by increased HPA axis activity in response to acute stress (Romeo et al., 2006a). In humans, there are increases in basal activity, as well as enhanced HPA axis stress reactivity during adolescence (Gunnar et al., 2009), which may place adolescents at higher risk for psychopathology. This may explain the increasing incidences of psychiatric disorders, such as anxiety and mood disorders, eating disorders, personality disorders and substance abuse that arise during adolescence (Gunnar and Quevedo, 2007, Paus et al., 2008). In rats, similar observations have been made, where adolescent rats exposed to stressors (e.g. hypoxia, foot shock, restraint) have prolonged plasma ACTH and corticosterone responses that last twice as long as those observed in adults (Romeo et al., 2006b, Goldman et al., 1973).

Furthermore, although sex differences in neuroanatomy and circuitry exist before the onset of puberty, the sex difference in risk for mood disorders rise sharply after puberty (e.g. after puberty, incidences of major depression are two times higher in females compared to males until after menopause) (Altemus et al., 2014). Rat studies show that the blunting of the male HPA stress axis occurs during puberty, due to the rise in testosterone and its interaction with the "organised" brain changes induced by neonatal testosterone (section 1.3.4) (Gomez et al., 2004). Differences in neuroactive steroid modulation of anxious behaviour also seem to appear during puberty, where it has been reported allopregnanolone triggers anxiety in female adolescent mice, while it decreases anxiety in adult female mice (Shen et al., 2007). In females, reproductive events throughout the life course such as the oestrous cycle, pregnancy and also menopause, can all dramatically alter the responsivity to stress. Apart from changes during the oestrous cycle, where varying oestrogen and progesterone level can affect ACTH levels, observed in rat studies (Viau and Meaney, 1991), the HPA axis undergoes dramatic adaptations during reproductive events such as pregnancy and lactation, where there is in general dampening of the HPA axis activity, which will be explained further in section 1.4 (Brunton and Russell, 2008b, Brunton et al., 2008).

Lastly, the HPA axis tone also increases with age, where there is hyperactivity during old age (Herman et al., 2001). Rat studies have attributed these changes to central mechanisms such as an increase in extracellular glutamate release in the hippocampus and prefrontal cortex following stress (Lowy et al., 1995), while other studies summarised in the following reviews have suggested increased local amplification of glucocorticoid action (Yau and Seckl, 2012), or a decrease in negative feedback inhibition due to decreased GR in the hippocampus (Sapolsky et al., 1986). In humans, aging is associated with increased cortisol, and this pattern seems to be more pronounced in females, again attesting to the sex differences that are present (Larsson et al., 2009, Lavretsky and Newhouse, 2012).

1.3.6 Neuroactive steroids

As briefly introduced in section 1.3.2.3, neuronal signalling can be augmented by steroids in the brain. These steroids were first termed "neurosteroids" by Baulieu, referring to steroids produced *de novo* in the brain (Baulieu, 1991). However, a broader term "neuroactive steroids" is now used to describe these steroids,

described by Paul and Purdy in 1992 as “endogenous or synthetic steroids which, irrespective of their origin, can rapidly alter the excitability of neurones via actions on membrane-bound receptors in the brain” (Paul and Purdy, 1992). Endogenous neuroactive steroids can be synthesised in the periphery (e.g. in the gonads or adrenal glands) or *de novo* in the brain.

In this thesis, the term “neuroactive steroid” will be used to refer to all steroids found in the brain that are able to regulate neural functions rapidly, regardless of their production site (periphery or in the brain) or the receptors they signal through (i.e. the mechanism of action of “neuroactive steroids” does not merely have to be restricted to modulating neurotransmitter signalling, as they can act through steroid nuclear receptors as well) (Melcangi et al., 2008).

1.3.6.1 Molecular actions of neuroactive steroids

Neuroactive steroids can rapidly modulate GABA (inhibitory) and glutamatergic (excitatory) signalling, by binding allosterically to the $GABA_A$ receptor and the N-methyl-d-aspartic acid (NMDA) glutamate receptors (Rupprecht, 2003), therefore can exert substantial acute modulatory actions on the activation or inhibition of the HPA axis (Myers et al., 2014, Herman et al., 2004, Gunn et al., 2011).

$GABA_A$ receptors are pentameric, made up of two α subunits (which can be formed from subunits α1-α6), two β subunits (which can be formed from subunits β1-3) and one γ subunit (from γ1-3) (Fig 1.5) (Enna, 2007). There is considerable diversity in the subunit make-up and regional distribution of $GABA_A$ receptors allowing for differential interactions with neuroactive steroids (Belelli et al., 2002), and the control of different behaviours (Wang, 2011, Sieghart and Sperk, 2002). The most common receptor combination within the CNS is the α1β2γ2 configuration (60% of all $GABA_A$ receptors; which also mediates sedative and anticonvulsant activity), followed by the α2β3γ2 configuration (15–20%, which also mediates anxiolytic activity) (Möhler et al., 2004). 3α,5α-reduced metabolites of DOC, progesterone and testosterone bind to a site distinct from the GABA binding region, opening the ion channel and allowing for the enhanced influx of Cl^- ions by increasing both channel frequency and channel opening duration, leading to sustained hyperpolarisation of the neurone, eventually resulting in increased inhibition of neural activity (Fig 1.5) (Lambert et al., 2001, Majewska et al., 1986, Morrow et al., 1987, Akk et al., 2007).

NMDA receptors assemble as tetrameric complexes, with two GluN1 subunits coupled with either GluN2/GluN2 or GluN2/GluN3, forming a channel through which the flow of positive ions is permitted (Fig 1.5) (Traynelis et al., 2010). Similar to the GABA receptors, there is diversity in NMDA receptors subtypes, where different subunit combinations formed from eight GluN1 subtypes, two GluN2 subtypes and two GluN3 subtypes can lead to different functional characteristics (Cull-Candy et al., 2001, Malayev et al., 2002). Neuroactive steroids such as pregnenolone sulfate (PregS) (Wu et al., 1991) and dehydroepiandrosterone (DHEA) (Baulieu and Robel, 1998) tend to positively modulate NMDA receptors, whilst 17β-oestradiol is a negative modulator of the NMDA receptor (Weaver et al., 1997). These steroids act together to regulate NMDA activity by altering the channel opening time and receptor sensitivity (Traynelis et al., 2010), preventing NMDA receptor over-activation which can result in excitotoxic cell death (Hilton et al., 2006). Apart from modulating stress-responsive circuits, clinically, NMDA receptors are known to be also involved in modulating neuronal plasticity, and an enhancement of NMDA receptor function has been linked the improvement of outcomes of cognitive decline (Collingridge et al., 2013), as well as the alleviation of the negative symptoms of schizophrenia (Marx et al., 2011).

In addition to the NMDA and GABA receptor, other receptors that can be modulated by the rapid action of steroids include the α-amino-3-hydroxy-5-methylisoxazole propionate (AMPA) receptor, sigma-1 receptors, nicotinic, muscarinic, kainate, serotonergic and glycine receptors (Zheng, 2009). Sex steroids for instance, seem to also have effects on the brain serotonin system, where 17β-estradiol, testosterone and progesterone act as antagonists at the 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) receptor, which are linked to the regulation of mood and anxiety (Rupprecht, 2003, Birzniece et al., 2006). Sex steroids can also exert rapid signalling actions through binding to their cognate receptors in a non-genomic manner (Wierman, 2007). In fact, their 5α-metabolites, such as dihydroprogesterone (5α-DHP) and 5α-dihydrotestosterone (5α-DHT), have also been found to be able to bind to the progesterone receptor and androgen receptor respectively to exert rapid effects (Rupprecht et al., 1993, Foradori et al., 2008).

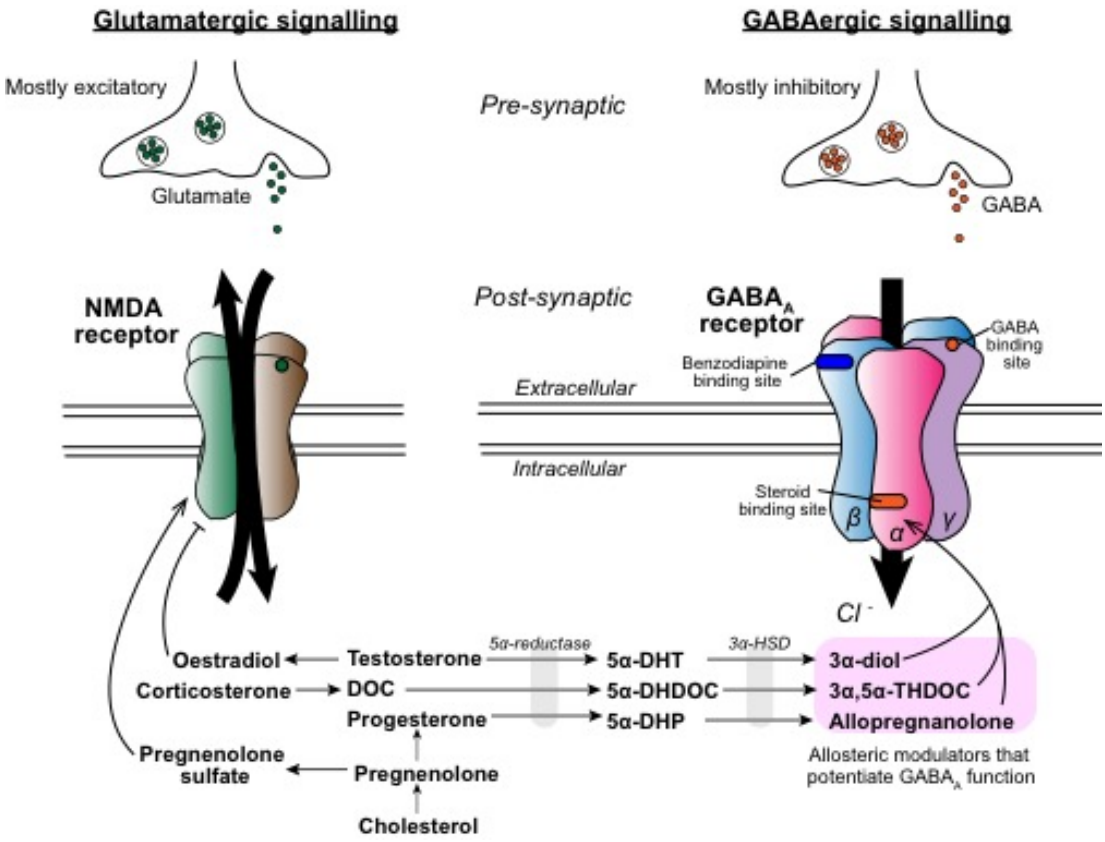


Figure 1.5 Examples of neuroactive steroid regulation of neuronal signalling. Neuroactive steroids can impact both excitatory signalling and GABAergic signalling. The $GABA_A$ receptor contains different ligand binding sites for GABA, benzodiazepine, barbiturate and steroids.

1.3.6.2 Neuroactive steroids and stress responses

Acute stress results in the robust increase of neuroactive steroid production, especially allopregnanolone and THDOC, when investigated in the rat brain (Purdy et al., 1991). Given their potentiating effect on GABA-mediated neuronal inhibition, their increase has been proposed to constitute a negative feedback mechanism to terminate the HPA axis (Gunn et al., 2015, Wirth, 2011). This is evident from experiments which show that the pre-treatment of either allopregnanolone or THDOC significantly attenuates the stress-induced elevation of plasma ACTH and corticosterone in rats, possibly through the inhibition of CRH-producing neurones at the mpPVN (Owens et al., 1992, Patchev et al., 1996). However, it has also been reported in adult mice that the increase in THDOC is necessary for the mounting an HPA axis stress response (Sarkar et al., 2011).

In cases of chronic stress, altered baseline neuroactive steroid concentrations can be observed, which may represent increased allostatic load that can underlie HPA axis abnormalities and eventually lead to disease (e.g. anxiety/depressive-like phenotypes) (Evans et al., 2012). HPA axis dysregulation in chronic stress has been associated with an imbalance between excitatory and inhibitory signals, where there is generally increased excitatory transmission (Flak et al., 2009) and decreased inhibitory transmission (Verkuyl et al., 2004) in the PVN of rats. Allopregnanolone and THDOC may contribute to the imbalance, as both their brain levels are found to be decreased following chronic stress in mice and rats (Dong et al., 2001, Serra et al., 2000). 5α-reductase type 1 expression was also reported to be decreased in glutamatergic neurons of the FC and BLA following chronic stress in a separate study in mice (Agis-Balboa et al., 2007). In humans, levels of endogenous 3α-reduced neuroactive steroids in plasma and cerebrospinal fluid are reported to be decreased in patients with stress-related affective disorders, while elevated neuroactive steroid levels are associated with anti-depressant actions (Uzunova et al., 2006). In rodent models of psychiatric diseases, exogenous administration of THDOC and allopregnanolone brings about potent anxiolytic actions in anxiety models (Bitran et al., 1995, Engin and Treit, 2007) and antidepressant-like actions in models of depression (Pinna et al., 2009).

Given the role of neuroactive steroids in rapidly regulating stress responses, changes in the levels of neuroactive steroids in the brain can therefore be informative in the understanding of molecular basis of differences in stress responses, such as in different sexes, or in stress-related disorders. The development of a reliable method for the quantification of neuroactive steroids in the brain is therefore endeavoured in this thesis, which will be elaborated in Chapter 3. The panel of steroids to be quantified are chosen based on their involvement in the modulation of the stress response. They include corticosterone (as final output of the HPA axis) and its inactive metabolite 11-DHC, the $GABA_A$ modulators allopregnanolone and THDOC and their precurors (DHP and progesterone, and DHDOC and DOC, respectively). Additionally, gonadal hormones such as estradiol and testosterone and its metabolites are also included, as these hormones are known to modulate the sex differences in stress responses.

1.4 What happens during pregnancy?

Pregnancy is a dynamic period marked by drastic physiological changes in both the mother and the foetus (Brunton et al., 2008). These changes include alterations to the cardiovascular, immune, and metabolic systems of the mother, which are necessary for the proper growth and the development of the foetus, as well as to prepare for parturition and lactation (Thornburg et al., 2015). These adaptations are mediated by a host of different hormonal signals, which can originate from the placenta, maternal or foetal endocrine organs, allowing for cross-talk and interactions between the maternal-placental-foetal triad. It is also of note that endocrine interrelationships between the mother, placenta, and foetus show species diversity, and are different in rodents and in humans (Albrecht and Pepe, 2015).

1.4.1 Mother

In the context of this thesis, one of the important adaptations during pregnancy (especially late pregnancy) is that the responsiveness of the maternal HPA axis is greatly reduced, following observations in rat and mouse studies (Neumann et al., 1998, Douglas et al., 2003). Given that the foetus is sensitive to the effects of glucocorticoids, a reduced maternal stress response system provides a first line of defense to prevent excessive foetal exposure to maternal glucocorticoids. Alteration of glucocorticoid levels may also aid immune adaptations in the mother, which ensures that the foetus is not rejected (Mor and Cardenas, 2010, Arck et al., 2007) and metabolic changes to ensure enough nutrients are being provided for the optimal development of physiological systems in the growing foetus (Welberg and Seckl, 2001). The mechanisms of these HPA axis adaptations are elaborated below with observations from both rat and human pregnancies.

1.4.1.1 Maternal basal HPA axis activity

In pregnant rats, basal concentrations of circulating corticosterone are elevated, with a gradual increase from mid-pregnancy to late-pregnancy (Waddell and Atkinson, 1994). Whilst the circadian variation in corticosterone secretion in pregnant rats are still present, they become flattened due to the increased levels at the nadir of the circadian rhythm (Atkinson and Waddell, 1995). ACTH concentrations however, do not show a circadian rhythm and levels remain low in late pregnancy (Atkinson and Waddell, 1995). The increase in basal corticosterone in pregnant rat is therefore not likely to be due to central mechanisms, but can be attributed to increased sensitivity

of the adrenal gland to ACTH, mediated by oestrogen, as it has been shown in female rats that increasing circulating oestrogen increases adrenal sensitivity to ACTH (Figueiredo et al., 2007). However, a compensatory increase in CBG occurs as well, thus the total amount of free and unbound corticosterone seem to be decreased during pregnancy (Douglas et al., 2003).

In the maternal rat brain, the basal expression of CRH mRNA in the mpPVN is reduced (Johnstone et al., 2000), concomitant with reduced release of CRH into the median eminence (Ma et al., 2005), which may explain suppressed ACTH levels (Atkinson and Waddell, 1995). Additionally, glucocorticoid-mediated negative feedback control also seem to be enhanced. Firstly, there is evidence of a modest upregulation of GR mRNA expression in the hippocampal dentate gyrus during late pregnancy near parturition, which may promote negative feedback processes via GR binding (Johnstone et al., 2000). Secondly, the activity of 11β-HSD1 (which reactivates corticosterone from 11-DHC) is increased in the PVN and anterior pituitary, which indicates increased local glucocorticoid availability and suggests enhanced negative feedback inhibition (Johnstone et al., 2000). Despite elevated peripheral corticosterone levels, these brain adaptations suggest that there is a reduced central drive of HPA axis activity (Brunton and Russell, 2015).

Similarly, in human pregnancies, patterns of increase also observed for plasma cortisol concentrations (Carr et al., 1981), concomitant with an oestrogen-mediated increase in CBG (Demey-Ponsart et al., 1982). Hypercortisolemia in human pregnancies however, is mainly driven by the placental production of CRH (elaborated in section 1.4.2), which increases the production of ACTH and cortisol in a manner that is autonomous and not subject to normal HPA axis negative feedback control (Carr et al., 1981, Jung et al., 2011, Wadhwa et al., 1997).

1.4.1.2 Maternal HPA axis responses to stress

Whilst the maternal HPA axis can be considered to be in a state of hypersecretion under basal conditions, the responsiveness of the HPA axis following stress is attenuated during late pregnancy. In the rat, HPA axis hyporesponsiveness, which begins in mid pregnancy, is maintained through parturition and lactation until the pups are weaned (Neumann et al., 1998, Wigger et al., 1999, Windle et al., 1997). Pregnant rats subjected to a psychological stressor such as the elevated plus maze (Neumann et al., 1998), a physical stressor such as immune challenge (Brunton et

al., 2005), or a combined physical and psychological stressor such as swim stress (Neumann et al., 1998, Douglas et al., 1998) all show reduced peak ACTH and corticosterone secretion.

Underlying mechanisms of HPA hyporesponsiveness include a decreased noradrenergic drive from the NTS, as no noradrenaline release from NTS neurones into the PVN is observed following physical stressors in a rat model (Douglas et al., 2005). This lack of noradrenaline release in the PVN, in turn, seems to be due to the inhibitory action of allopregnanolone at the NTS, via an endogenous opioid pathway (Brunton et al., 2005, Brunton et al., 2009).

Concentrations of allopregnanolone increase in both the maternal blood and brain during pregnancy, due to the increase in production of its precursor, progesterone (Concas et al., 1998). In late pregnant rats, inhibiting allopregnanolone production with the 5α-reductase inhibitor, finasteride, restores normal HPA axis responses to systemically administered IL-1β, while administering allopregnanolone to virgin rats mimics the HPA hyporesponsiveness observed in late pregnant rats (Brunton and Russell, 2008a). Apart from acting through potentiating $GABA_A$-mediated inhibitory networks in the brain (Patchev et al., 1996), allopregnanolone also exerts its effect on the HPA axis via an endogenous opioid pathway (Brunton et al., 2005).

Endogenous opioids can suppress HPA axis responsiveness in late pregnancy by acting on noradrenergic NTS neurones, preventing noradrenaline signalling to the PVN, reducing HPA axis activation and CRH production, and consequently decreasing ACTH and corticosterone production (Brunton et al., 2009). In pregnancy, there is an upregulation of central inhibitory opioid mechanisms, where the mRNA expression for the endogenous opioid, proenkephalin-A, and for μ-opioid receptor are increased in the NTS of the brainstem (Brunton et al., 2005). This increased inhibitory opioid tone seems to be induced by the increase in allopregnanolone, as a similar increase in opioid-related gene expression is observed when virgin females are treated with allopregnanolone (Brunton and Russell, 2008a). The mechanism by which allopregnanolone increases brainstem opioid expression has not been investigated, but may again involve rapid signalling through $GABA_A$ receptors (Brunton et al., 2012).

In pregnant women, the responsiveness of the HPA axis to stress has not been extensively studied like in the rat, but is known to be generally dampened as compared to non-pregnant females (Duthie and Reynolds, 2013, de Weerth and Buitelaar, 2005). Plasma ACTH and cortisol secretion have been observed to be suppressed in pregnant women in response to exogenous CRH administration (Schulte et al., 1990), whilst salivary cortisol is also reduced in pregnant women in response to a physical cold stress (Kammerer et al., 2002). Nonetheless, variation is observed, possibly due to the nature of different stressors, as salivary cortisol secretion was reported to be instead prolonged following a social stress test in pregnant women during the second trimester in pregnancy (Nierop et al., 2006).

1.4.2 Placenta

The placenta integrates and communicates environmental factors such as the nutritional status and stress status of the mother to the developing foetus by altering its vasculature, oxygen, nutrient transport, nutrient concentrations, and also by producing factors (e.g. hormones or growth factors) that may directly affect foetal growth and development (Albrecht and Pepe, 2015, Burton et al., 2016).

1.4.2.1 Structure of the placenta

The placenta is primarily of foetal origin, consisting mostly of foetal trophoblast cells, but has a maternal component in the decidua, which contains maternal arteries and immune cells (Sadovsky and Jansson, 2015). There are two structurally distinct regions of the foetal-derived trophoblastic component, the junctional zone (JZ) and the labyrinth zone (LB) (Fig 1.6). The JZ, which is located adjacent to the maternal decidua, contains spongiotrophoblast and trophoblast giant cells, the two major endocrine cells that contribute to the secretion of hormones (Ain et al., 2006, Hu and Cross, 2010). The JZ is devoid of foetal vessels and does not participate in the exchange of maternal and foetal materials (Fig 1.6). The LB on the other hand, consists of both maternal and foetal blood vessels, although the two circulations do not mix. The LB is considered the site of foeto-maternal exchange, where foetal blood is separated from maternal blood sinuses by tree-like villi lined by syncytiotrophoblasts cells (Fig 1.6), thereby increasing the surface area for the exchange (Sadovsky and Jansson, 2015).

Given their different functions, gene and protein expression patterns of the different zones are expected to be quite different. Indeed, global gene analysis in the human placenta have found very different expression patterns among the foetal (mainly LB), maternal (basal plate in humans; equivalent to JZ in rats), and middle sections (mainly LB) of the placenta (Sood et al., 2006). A similar study using RNA sequencing of the rat placenta, reported unique expression patterns for each zone, characterised by biological processes specific to the zone. 4000 differentially expressed genes were observed between the zones, and genes related to transport and vasculature-related processes predominated in the LB, while genes related to hormone secretion were more highly expressed in the JZ (Shankar et al., 2012).

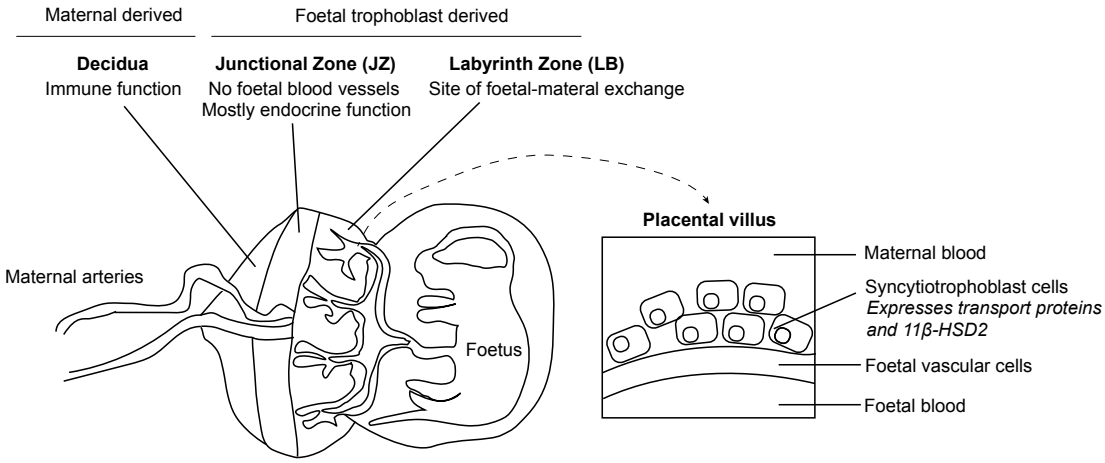


Figure 1.6: Structure of the rat placenta. Different zones of the placenta have different functions. The maternally-derived decidua is important in regulating the mother's immune relationship with foetus, while JZ secretes hormones mainly into the maternal circulation. The LB contains both foetal and maternal blood vessels and is the site of foetal-maternal exchange of gases, nutrients and other materials. 11β-HSD2 for instance, is expresses in syncytiotrophoblast cells in the LB. Adapted from Bronson and Bale, 2016.

1.4.2.2 Function of the placenta

The placenta has three basic functions: (i) to facilitate gas exchange and nutrient transfer; (ii) to act as an endocrine organ to ensure the viability of the pregnancy, and (iii) to act as a protective barrier (Griffiths and Campbell, 2015, Burton et al., 2016).

Transfer of gases and nutrients: Both oxygen and carbon dioxide readily diffuse across the placenta by passive diffusion, where oxygen is transferred from the

maternal blood to the foetal blood, while foetal blood releases carbon dioxide back into the maternal circulation. Specific nutrient transporters expressed in the syncytiotrophoblast cells of the LB facilitate the transport of glucose (e.g. via the GLUT1 transporter), amino acids and fatty acids across the placenta from mother to foetus (Sadovsky and Jansson, 2015).

Endocrine organ: The placenta is an endocrine organ and produces a wide array of hormones, including those from the prolactin-growth hormone family (e.g. prolactin, placental lactogen and growth hormone), steroid hormones (oestrogens and progesterone) and neuropeptides (serotonin, melatonin and oxytocin), reviewed in both human and rodent studies in (Napso et al., 2018). Progesterone for instance, is an important pregnancy hormone and is essential to the viability of the foetus, as it maintains the endometrium and suppresses uterine contractions which would invariably terminate the pregnancy. In humans, the syncytiotrophoblasts cells of the placenta takes over the synthesis of progesterone from the corpus luteum at approximately 8 weeks of pregnancy (Albrecht and Pepe, 2015). In rodents however, the placenta produces neglible amounts of progesterone, but produces signals (e.g. placental lactogen) that maintains corpus luteal progesterone production (Galosy and Talamantes, 1995, Carter, 2012). Apart from physically maintaining the pregnancy, progesterone alters the maternal physiology in order to accommodate the pregnancy, for instance, by altering the immune system to prevent the rejection of the foetus in all mammalian species (Arck et al., 2007). The placenta expresses steroidogenic enzymes, although the expression pattern of the enzymes depends on the stage of pregnancy, as well as the species (Strauss et al., 1996). It is known that steroidogenic enzymes 5α-reductase (both isotypes 1 and 2) and 3α-HSD are expressed in the human and sheep placenta, which contributes to the production of allopregnanolone (Hirst et al., 2009), Allopregnanolone not only modulates the maternal HPA axis during pregnancy, but also plays a role in regulating foetal brain development (reviewed in (Brunton et al., 2014)).

Protective function: As the maternal and foetal circulations do not mix, the syncytiotrophoblasts cells of the placenta acts as a physical barrier against xenobiotic substances, but also mediates the transfer of immunoglobins such as IgG to provide the foetal compartment with some form of immune protection (Simister, 2003). 11β-HSD2 is also expressed in the placenta, which limits the amount of active maternal glucocorticoids than can be transferred into the foetal circulation

(Brown et al., 1996, Chapman et al., 2013). This adaptation represents the second line of defence (in addition to the dampening of the maternal stress response) against excessive glucocorticoid exposure of the foetus, following a stressful encounter. 11β-HSD2 expression is dynamic and changes according to the pregnancy stage, which will be elaborated in Chapter 5.

Given these important functions, the placenta may therefore play a central role in mediating foetal programming as any perturbations to its normal function could lead to compromises in foetal growth and development (Sferruzzi-Perri and Camm, 2016, Kratimenos and Penn, 2019, Bronson and Bale, 2016). The role of the placenta will be further investigated in Chapter 5 and Chapter 6.

1.4.2.3 Species differences in the placenta

As a rat model is used in this thesis, differences between the rat and human placenta will be briefly discussed. Although mammalian placentation is largely conserved, differences in the structure and function of rodent versus human placentas exist, and many are also related to the inherent differences between rodent and human pregnancies (e.g. different gestational length, number of offspring per pregnancy) (Soares et al., 2012, Furukawa et al., 2014, Ilekis et al., 2016, Grigsby, 2016). In terms of its role as a endocrine organ, certain key differences arise, especially in the expression patterns of enzymes needed to synthesise hormones in the rat and the human placenta.

Firstly, the human placenta secretes large amounts of CRH into the maternal circulation, whilst the rat placenta does not produce CRH at all (Robinson et al., 1988, Heussner et al., 2016). Placental CRH secretion increases the production of ACTH and cortisol, but unlike the hypothalamus, cortisol in the placenta acts in a positive feedback loop to further augment the secretion of placental CRH (Majzoub and Karalis, 1999, Robinson et al., 1988). Placental CRH can thus further regulate processes like the timing of parturition, stress responses, cortisol-mediated metabolic actions and energetics (Gangestad et al., 2012) in pregnant women in a manner that is not observed in rats (Alcántara-Alonso et al., 2017).

Additionally, although progesterone and oestrogens are secreted in large amounts during both rat and human pregnancies, the contribution of the placenta in secreting

these hormones differ between the two species (e.g. the rat placenta does not produce large amounts of progesterone, mentioned in 1.4.2.2). The rat placenta also does not express aromatase, but instead provides androgenic precursors (e.g. androstenedione) for oestradiol production by the maternal ovary (Jackson and Albrecht, 1985). On the other hand, in humans, the placenta expresses aromatase to convert testosterone into oestradiol, but lacks 17α-hydroxylase to synthesise androstenedione, and is thus dependent on the maternal and foetal adrenal glands to generate androgenic precursors (see Fig 1.3 for steroid conversion diagram) (Strauss et al., 1996). For instance, the human foetal adrenal gland produces a majority of the DHEA that the placenta uses as a precursor to make androstenedione (via the action of 3β-HSD), then subsequently testosterone and oestrogens (Warren and Timberlake, 1964). These findings also demonstrate how the placenta in general is dependent on both the maternal and foetal compartments for proper functioning, but the specific interactions are different between rats and humans. Nonetheless, despite these differences, the rat model is still helpful in its ability to allow for manipulations, as long as these differences are kept in mind (Bonney, 2013).

1.4.2.4 Sex differences in the placenta

Sex differences occur not only in the foetus, but also in the placenta (Alur, 2019, Clifton, 2010). The male and female placenta are inherently different as they are derived from foetal cells, therefore would have unequal expression of X- and Y-linked genes (Gabory et al., 2013). In human placentae, apart from sex-linked genes, there are also clear sex differences in the expression of genes related to immune or growth pathways, where they are generally expressed at higher levels in females than in males (Sood et al., 2006).

Prenatal stress is known to exert unequal influences on male and female offspring and the varying vulnerabilities have been proposed to be mediated by the placenta during foetal development (Bale, 2016, Rosenfeld, 2015). Indeed, male and female placenta seem to respond differently to excess glucocorticoids in mice (O'Connell et al., 2011, Cuffe et al., 2017b) and in humans (Stark et al., 2009), indicating that they employ different strategies to ensure foetal growth and survival in the face of homeostatic perturbations (Clifton, 2010). In human pregnancies, it seems that male

foetuses seem to be more affected by *in utero* compromises (Eriksson et al., 2010, Edwards et al., 2000, Kalisch-Smith et al., 2017). In later life, a sex bias is also observed for neurodevelopmental disorders such as autism spectrum disorder, anxiety and schizophrenia, where males often have a higher disease risk (Bale, 2011, Baron-Cohen et al., 2011). In mice, expression of placental O-linked N-acetylglucosamine transferase (OGT), which can modulate glucocorticoid responses, are also found to be twice as high in the female placenta (Pantaleon et al., 2017), and can be affected in a sex-dependent manner following stress (Howerton and Bale, 2014). The implication of this is that in studying foetal programming, the consideration of sex need to apply to the placentae as well, in addition to the foetus. Moreover, male and female placentae, arising from the same pregnancy, should not be assumed to be equivalent.

1.4.2.5 Oxidative stress in the placenta

The placenta is characterised by extremely fast growth, and placentation itself is an invasive process that involves cell proliferation, differentiation and the restructuring of blood vessels (Soares et al., 2014). In rats for instance, a fast growth of the placenta from 1–2 mm in diameter at GD4 to 21–23 mm at GD21 can be observed (de Rijk et al., 2002); while in humans the growth of the placenta is precocious especially in the first trimester of the pregnancy (Burton and Jauniaux, 2018). The increased exchange of materials and secretion of hormones in order to meet the demands of the pregnancy also mean that the metabolic activity of the placenta is extremely high. The outcome of this high metabolism is the generation of large amounts of reactive oxygen species (ROS). The placenta is therefore considered to be in a constant state of mild oxidative stress (Myatt and Cui, 2004, Wu et al., 2016), which may predispose it to dysfunction in the event of additional environmental stressors. This concept will be further developed in Chapter 6.

1.4.3 Foetus

Foetal growth and development is a complex process determined by the foetal genome and is influenced by environmental factors (e.g. the availability of nutrients and oxygen to the foetus, maternal nutrition and stress). The process is orchestrated by various growth factors and hormones of maternal, foetal and placental origin (Murphy et al., 2006). Hormones such as insulin, insulin-like growth factors (IGF) and glucocorticoids act as maturational signals *in utero*, directing foetal metabolism,

tissue development and differentiation according to the prevailing intrauterine conditions (Gicquel and Le Bouc, 2006, Fowden and Forhead, 2009). During late pregnancy, there is also a glucocorticoid surge in foetuses which promotes organ maturation, especially lung maturation, in preparation for extrauterine life (Venihaki et al., 2000, Fowden et al., 1998). In rats, this surge is contributed by both production of corticosterone from the foetal adrenal gland (Dupouy et al., 1975, Milkovic et al., 1973b), as well as changes in the placenta.

Within the foetal brain itself, cellular processes involved in the development of the nervous system include proliferation, migration, neuronal differentiation, synaptogenesis, myelination and apoptosis of neurones, also involving the glial cells (i.e. microglia, astrocytes, and oligodendrocytes) (Rice and Barone, 2000, Stiles and Jernigan, 2010). Neurotransmitters are expressed in the nervous system at a very early stage of embryogenesis, and in comparison to their role in synaptic transmission in adulthood, during foetal life they act instead as morphogens that facilitate the growth of neurones, mediating the development of these neurotransmitters systems (Herlenius and Lagercrantz, 2004, Rice and Barone, 2000). Other morphogens include growth factors and steroid hormones also act in this narrow temporal window to ensure that brain regions are correctly organised and connected. Disruption of any aspect of this precisely orchestrated and pre-determined developmental trajectory may result in lasting organisational changes, acting as the precursor for disrupted brain function and behaviour later in life.

The specific role of steroids in foetal development, especially in late pregnancy in the rat (and a few other animal models), will be thoroughly reviewed and examined in Chapter 5. Despite differences between the rodent and human brain, analogous structures which are responsible for sensory, motor and cognitive functions can be identified (Semple et al., 2013). The sequence of neural development is comparable among the two species, although processes occur on considerably different time scales (Bayer et al., 1993).

Although the placenta and maternal steroid hormones play a role in controlling foetal growth, it should be emphasised that the foetus itself has an independent capacity to modulate its own development, and also to further modulate signals from the placenta. For instance, sex steroids and glucocorticoids are produced by the foetal gonads and adrenal glands. In the rat foetal brain and liver, 11β-HSD enzymes are

also expressed to control local concentrations of glucocorticoids (Diaz et al., 1998). Therefore, the foetal compartment should not be viewed as merely an acquiescent receiver of environmental signals from the maternal and placental component, but also an active contributor in the shaping of the pregnancy and its own growth and development.

1.5 Gestational stress

The physiological response to an acute stressor was introduced in section 1.2.1, and it was recognised in section 1.2.2 that chronic or traumatic stressors can cause allostatic overload, leading to pathology. Section 1.4 then briefly introduced the alterations in maternal physiology to ensure a successful pregnancy. These pregnancy-specific changes (e.g. hypoactivation of the maternal stress response, development of the placenta) therefore becomes the new "physiological norm". Any deviation from this "physiological norm", for instance by constant or prolonged stress, may culminate in the dysregulation of neuroendocrine systems, leading to compromised pregnancy outcomes (Lobel et al., 2008, Coussons-Read, 2013). These outcomes can affect both the mother and the developing child, and can range from immediate effects that endanger the pregnancy or health of the mother (e.g. preterm birth, miscarriage or gestational diabetes/hypertension), to more subtle compromises that may only present themselves later in life (e.g. postnatal depression in the mother, or a risk for mood disorders in the offspring in adulthood) (Coussons-Read, 2013, Sandman et al., 2012, Wadhwa, 2005).

In this section, the DOHaD concept is revisited again, and the effects of gestational stress on offspring outcomes in later life will be briefly summarised, with a particular focus on the rodent prenatal social stress model utilised in this thesis. Known or proposed mechanisms mediating these negative outcomes are then covered, focusing on two aspects: (i) mechanisms underlying the "programmed" behavioural phenotypes in the adult offspring, and (ii) mechanisms underlying how the stress is being transmitted from mother to foetus (i.e. foetal "programming").

1.5.1 Modelling gestational stress in the rat

Gestational stress in humans:

Gestational stress in humans can involve many different kinds of stressors that range from malnutrition, mental and metabolic disorders, to the exposure to

environmental toxins (Lefmann and Combs-Orme, 2014, Coussons-Read, 2013). Studies in the past have relied upon data collected from populations experiencing natural or man-made disasters such as periods of famine, extreme weather conditions, or war (Lumey et al., 2011, Liu et al., 2016a, King and Laplante, 2005). However, these events tend to be a rare occurrence, and the form of chronic stressors that the majority of pregnant women face on a day-to-day basis are far subtler, are inherently more complex, and additionally have various social and psychological components (Epel et al., 2018). Questionnaires have thus been designed to assess these forms of "pregnancy-specific stress", which are recognised as a separate entity from distress faced by normal populations, owing to different physiology and also the social circumstances surrounding it (Lobel et al., 2008). They include stress arising from physiological changes, relationship strains, anxiety about labour and delivery, and concerns about the baby's health etc. (Lobel et al., 2008, Dunkel Schetter, 2011). Physiological indices such as cortisol, blood pressure or heart rate reactivity are often also used as correlates of maternal stress (De Weerth et al., 2007, Entringer et al., 2010). Nonetheless, due to ethical concerns that render it difficult to manipulate stressful conditions in humans, these studies are generally correlative and observational in nature, and the causal mechanisms underlying how offspring programming occurs remain largely unclear (Dipietro, 2012).

Animal models of gestational stress:

The molecular and cellular mechanisms altered by gestational stress can be better studied using animal models, which allows for experimental manipulation of the type, magnitude, and extent of the gestational stress. Animal models that have been used include rodents, with rats generally more popular a model than mice (Ellenbroek and Youn, 2016), as well as sheep (Barry and Anthony, 2008, Yawno et al., 2007), guinea pigs (Morrison et al., 2018, Kapoor and Matthews, 2005), pigs (Jarvis et al., 2006) and non-human primates (Schneider et al., 2001, Meyer and Hamel, 2014).

Immobilisation or restraint stress, ranging from a duration of 30 min to 6 hr, has been the most popular stressor used in rodents (Buynitsky and Mostofsky, 2009). In fact, one of the first studies regarding prenatal stress in rodents was carried out before the clinical DOHaD studies, in the 1970s where prenatal restraint stress was

used to study sexual differentiation of the rat foetus (Ward, 1972). Since then, it has been favoured by many groups due to its simplicity and also because it allows for the easy collection of blood samples from the immobilised dam to track hormonal changes across the stressful period. Over the years, other types of experimental models of stress have been used in prenatal stress research, including exposure to adverse environments (e.g. cold stress, hypoxic environment), fear-based stressors (e.g. predator odour, noise stress), or social stressors (Patchev and Patchev, 2006). Chronic variable stress, where the pregnant rat is exposed to a combination of different stressors randomly, is also commonly used by many groups, as the unpredictable nature of the stressor circumvents habituation of the stress response (Koenig et al., 2005).

Prenatal social stress model in rat:

In this thesis, maternal social stress was used as the stress paradigm, as it has good translational value and can better reflect the stressors likely to be encountered by pregnant women (e.g. bullying, lack of social support, social pressures or instability, domestic abuse) (Dunkel Schetter, 2011). In animal studies, social stress during pregnancy has been found to have effects in a range of other non-human mammals such as rats, mice, voles and monkeys (Kaiser and Sachser, 2005). Social stress is defined as stress that is elicited by a conspecific, and examples include social defeat, social submissiveness, establishment of a social hierarchy, aggressiveness or overcrowding (Pryce and Fuchs, 2017).

Social defeat via the resident-intruder paradigm is one way to induce social stress and it is usually applied to male rodents (Koolhaas et al., 2013). As female rats are not inherently aggressive, in order to apply this paradigm to female rats, lactating female rats nursing PND2-8 pups are used as “residents”, as they tend to strongly defend the home cage when encountered with an intruder (Rosenblatt et al., 1994, Bosch et al., 2004). In this modified resident-intruder paradigm, pregnant “intruder” rats were placed into the home cage of lactating “residents” for 10 min per day on GD16-20 (Brunton and Russell, 2010) (Chapter 2). Each specific lactating “resident” is also only exposed to the same “intruder” once to prevent habituation of responses in both the residents and intruders.

1.5.2 Effects of chronic gestational stress on offspring outcomes in later life

1.5.2.1 Human studies:

Empirical evidence from epidemiological studies linking chronic gestational stress to poor offspring outcomes are the foundations on which the DOHaD model was built on. In summary, gestational stress can result in negative impacts for the offspring across the life course, beginning in infanthood (e.g. difficult temperament), persisting through childhood and adolescence (e.g. attention-deficit hyperactive disorder, emotional problems, conduct disorder, learning difficulties, higher risk of autism spectrum disorder), and into adulthood (higher risk of schizophrenia, depression and anxiety) (Van den Bergh et al., 2017, Coussons-Read, 2013, Graignic-Philippe et al., 2014, Glover et al., 2010). It has been proposed that many of these conditions have underlying HPA axis dysregulation, assessed by measuring salivary or plasma cortisol and ACTH, either basally or in response to a mild stressor (e.g. the Trier Social Stress Test) (Glover et al., 2010, Entringer et al., 2009). It is however, not known if neuroactive steroids could underlie the observed HPA axis dysregulation. Alterations of immune function, increased incidence of asthma and allergy, and increased risk for metabolic disorders such as diabetes and obesity have also been reported (Entringer et al., 2012, Flanigan et al., 2016).

Alongside behavioural alterations, imaging studies also reveal that offspring of anxious mothers also exhibit changes in brain structure and function during childhood and adolescence (Buss et al., 2010, Mennes et al., 2009, Charil et al., 2010). On a molecular level, there is evidence of epigenetic changes in the offspring (Palma-Gudiel et al., 2015), as well as changes in telomere biology, where prenatally stressed children show lower birth weight and have shorter leukocyte telomere length (Entringer et al., 2011). In general populations of patients with mood disorders, there is evidence that basal plasma neuroactive steroid concentrations are altered, in pathological disorders such as gestational depression and premenstrual dysphoric disorder (Girdler and Klatzkin, 2007, Hellgren et al., 2014), although the relationship between these disorders and prenatal stress were not investigated in those same populations.

The mechanisms underlying the increased general susceptibility to psychopathology in prenatally stressed offspring also have not been fully elucidated. Even though children of gestationally stressed parents may show increased risk, they may be

affected in different ways, or some may not be affected at all, and these observations are likely to be confounded by other factors like gene-environment interactions or social factors (O'Donnell et al., 2014, Glover et al., 2010). Casual links between maternal stress and specific outcomes can therefore be better studied using animal models, where such factors can be better controlled.

1.5.2.2 Animal models:

In animal models, the evidence that prenatal stress can lead to deleterious effects in the offspring has also been unequivocal. The wide-ranging effects of prenatal stress in the rat model have been summarised by many researchers (Weinstock, 2017, Brunton, 2013, Boersma and Tamashiro, 2015). Several reviews have also discussed animal studies alongside human clinical studies, where many parallels in terms of outcomes can be drawn (Bock et al., 2015, Kofman, 2002). Similar to that observed in human populations, adult offspring from prenatal stress animal models show behavioural phenotypes such as heightened anxiety-like behaviours, enhanced or prolonged stress responses, greater occurrence of depressive-like behaviours, learning and memory deficits and altered social interactions to name a few (Weinstock, 2017).

Dysregulated HPA axis responses to acute stress, especially, have been reported in prenatally stressed offspring using different maternal stress paradigms (McCormick et al., 1995, Vallee et al., 1997, Henry et al., 1994, Maccari et al., 1995, Darnaudery and Maccari, 2008). In the brain, several changes underlie the PNS phenotype, including modifications of dendritic architecture or neuronal connectivity, changes in receptor subunit expression, or alterations in levels of neurotrophic factors (Boersma and Tamashiro, 2015, Weinstock, 2017). Metabolic outcomes of prenatal stress, such as diet-induced obesity, hypertension, cardiac dysfunction, and insulin resistance are also common (for reviews see (Vickers, 2011, Bertram and Hanson, 2001, Marciniak et al., 2017).

In these studies, striking sex differences are often also observed (Weinstock, 2007, Bale, 2011). Studies suggest that prenatally stressed male rats are more susceptible to various forms of psychopathology (Cheong et al., 2016, Van den Hove et al., 2013), however, this is not always the case, especially when only a few groups have directly compared the effect of prenatal stress in males versus females using similar testing and assessment paradigms (Weinstock, 2017).

Similarly, it is worth noting that there has also been considerable variability in terms of the nature of the outcomes, despite animal models (especially inbred rodent strains) having very similar genetic make-up. Even the same stress exposure carried out in different laboratories can result in different offspring effects, whilst morphological changes in the brain may not always be correlated to the same behavioural outputs (Weinstock, 2017), For instance, even when an identical stressor was used in Sprague-Dawley rats, one group found an increase in anxiety-like behaviour only in the male offsrping (Zuena et al., 2008), whereas others found an anxiety effect only in females (Schulz et al., 2011, Van den Hove et al., 2014). Stress responses are often very sensitive to even the smallest of changes in the environment, and various factors can affect outcomes, including the type and duration of stressor, the genetic background of the animal, the gestational period when stress is experienced, and postnatal influences, such as variations in maternal care (Weinstock, 2017, Boersma and Tamashiro, 2015). Given this inherent variability, the next section narrows down some of the factors that may cause variability, summarising the impact of prenatal stress from only the social stress model used in this thesis.

1.5.2.3 Rodent prenatal social stress model

The prenatal social stress model has been used to study the impacts of prenatal stress in the Brunton lab for several years, and a number of behavioural phenotypes and neurophysiological features have been characterised in the adult offspring (Brunton and Russell, 2010, Brunton and Russell, 2015, Grundwald et al., 2016, Lai, 2016). In summary, there is strong evidence for altered HPA axis function, increased anxiety-like behaviour, and learning and memory deficits in adult prenatally stressed (PNS) offspring, and these altered phenotypes show a distinct sex-specific expression pattern (Fig 1.7).

Effects of PNS on offspring response to acute stress and HPA axis function:

Both the male and female PNS offspring exhibit greater plasma ACTH and corticosterone responses to systemic IL-1β administration (a physical stressor that activates the immune system) or 30 min restraint stress (both psychogenic and physical stressor). The peak response is increased, indicating increased activation of the HPA axis, and responses are prolonged, possibly indicating impaired negative

feedback control and slower termination of the HPA axis (Brunton and Russell, 2010, Brunton et al., 2015). These are further elaborated in Chapter 4.

The greater ACTH and corticosterone in the circulation is accompanied by changes in the brain, which are also dependent on sex of the offspring. In PNS males, there was elevated CRH and AVP mRNA expression in the mpPVN is elevated and POMC expression in the anterior pituitary is increased compared to control; whilst in the PNS females, this was accompanied by only greater CRH mRNA expression in the PVN (Brunton and Russell, 2010). Nonetheless, this implies that there is increased excitatory drive to the HPA axis at the level of the PVN in both male and female PNS offspring. Underlying mechanisms for the increased drive remain unknown, although stronger activation of NTS neurones, which projects directly to the CRH-producing mpPVN neurones via noradrenergic signalling, is a possibility (Cunningham and Sawchenko, 1988, Rinaman, 2011).

Secondly, an imbalance in central MR and GR mRNA expression has been reported (Brunton and Russell, 2010, Lai, 2016), which suggests regulation of the HPA axis via negative feedback mechanisms could be impaired. MR mRNA expression is significantly reduced in the hippocampus of both male and female PNS offspring compared to same sex control offspring (Brunton and Russell, 2010, Lai, 2016). Although GR expression is largely unaffected in the PVN or hippocampus (Brunton and Russell, 2010), reduced GR expression is found in the medial prefrontal cortex (mPFC) in PNS offspring, which has a role to play in negative feedback inhibition of the HPA axis (Sullivan and Gratton, 2002, Lai, 2016). GR expression in the central amygdala (CeA) of male PNS offspring is also increased (Brunton and Russell, 2010), which is relevant as glucocorticoid signalling in the amygdala activates the HPA axis (Herman et al., 2005). GR signalling in the CeA may also mediate feed-forward effects within amygdala regions, leading to enhanced stress excitability and sustained CRH, ACTH and corticosterone release (Myers et al., 2012, Schulkin et al., 1998, Cook, 2002).

Thirdly, there is evidence that neuroactive steroid production may be compromised in PNS offspring, as lower 5α-reductase type 1 gene expression is found in some brain areas, such as the NTS (Brunton et al., 2015). Moreover, administration of $GABA_A$-modulatory steroids (e.g. allopregnanolone and 3β-androstanediol to female and male PNS offspring respectively) can rescue dysregulated HPA axis responses

to stress (Brunton et al., 2015), suggesting that deficient endogenous production of neuroactive steroids may underlie HPA axis hyperactivity in PNS offspring. How specific neuroactive steroids may act in discrete regions of the brain to affect the HPA axis will be further examined in Chapter 4.

Effects of PNS on anxiety and depression:

A sex-specific effect of prenatal social stress on anxiety-like behaviour in the offspring has been reported (Brunton and Russell, 2010). Only PNS males were affected, exhibiting more anxious-like behaviour than control offspring in both the light-dark box (LDB) and on the elevated plus maze (EPM), two tests commonly used to assess anxious behaviour in rodents (elaborated in Chapter 6).

The anxiety phenotype may be explained by changes in the CRH system in the amygdala, which is important for regulating anxiety-like behaviour (Smagin et al., 2001, Steimer, 2002). There are two major CRH systems in the brain, one in the PVN, and the other in the amygdala/BNST circuitry (reviewed in (Babaev et al., 2018)). An increase in CRH in the amygdala is usually associated with increased anxiety, as transgenic mice with CRH overexpression (van Gaalen et al., 2002, Stenzel-Poore et al., 1994) and rats which received lentiviral administration of CRH mRNA both show greater anxiety (Flandreau et al., 2012). Experiments from receptor knockout mice conclude that effects of CRH on CRH receptor type 1 (CRH-R1) promotes anxious behaviour (Smith et al., 1998), while action through the CRH receptor type 2 (CRH-R2) reduces anxiety behaviour (Kishimoto et al., 2000). In the PNS offspring of this rat social stress model, greater CRH-R1 mRNA expression is observed in the CeA and basolateral amygdala (BLA); while lower CRH-R2 mRNA expression is observed in the basomedial amygdala (BMA), which may explain the behavioural observations (Brunton et al., 2011). In female PNS rats, whilst no anxious phenotype is observed, there is increased CRH-R1 in the medial amygdala (MeA) and CRH-R2 in the BMA (Brunton et al., 2011).

Other than CRH, inhibitory neurotransmission via GABA also plays a key role in anxiety disorders. It is thus possible that alterations in GABA subunit expression, or concentrations of GABA modulators (e.g. neuroactive steroids) in the amygdala may contribute to the behavioural phenotype (Nuss, 2015, Babaev et al., 2018), although it has never been directly investigated in this model. One of the aims of this thesis is therefore to pursue these two lines of evidence (in Chapters 6 and 4, respectively).

Moreover, depression and anxiety are generally known to be co-morbid, and HPA axis dysregulation is generally also associated with a depressive phenotype, yet this has also not been investigated in this PNS model. Another aim of this thesis is thus to investigate whether depressive-like phenotypes are observed in the PNS offspring (Chapter 6).

Effect of PNS on learning and memory:

Male and female PNS offspring are differentially affected, depending on the memory task. Male PNS offspring show more deficits in spatial learning and memory tasks (Lai, 2016), whilst females are more affected for social memory-related tasks (Grundwald et al., 2016). The underlying mechanisms of these deficits also appear to be different.

Spatial learning and memory was tested using the Barnes maze, which consists of (i) a learning phase, where rats learn over the course of three days the most efficient way to locate an escape box in the maze, and (ii) retention tests, which are carried out after a period of time to examine memory retention and recall (Rosenfeld and Ferguson, 2014, Barnes, 1979). Both male and female PNS offspring do not appear to have spatial learning and memory deficits on the Barnes maze under basal conditions when compared to the respective controls (Lai, 2016). However, if male PNS rats are subjected to acute stress (using restraint) before the learning acquisition period, spatial learning and memory deficits are observed, to a greater extent than in control offspring (Lai, 2016). Male PNS rats thus show impaired spatial learning, but only under stressful conditions (Lai, 2016). However, as opposed to the effects of acute stress, pre-exposure to chronic stress (using a one-week variable stress regimen), instead improved spatial learning in PNS offspring on the Barnes maze, and this was more obvious in the male PNS rats than in the female PNS offspring. In terms of a sex difference in control rats, whilst control females rats outperformed control males in terms of behavioural flexibility on the Barnes maze without any stressors, these females instead show more deficits than the male controls when acute and chronic stressors are applied (Lai, 2016).

By using a specific MR antagonist, it was also found that the underlying mechanisms of this spatial learning deficit may be linked to decreased MR expression in the hippocampus (Lai, 2016, Brunton and Russell, 2010). In offspring which exhibited better performance on the Barnes maze following chronic stress,

there was also an accompanying elevation of MR hippocampal mRNA in the chronically stressed PNS animals (Lai, 2016). This additionally shows the complex interaction between prenatal stress, acute stress and chronic stress.

In the social memory test however, most of the deficits were observed in female PNS offspring and not males (Grundwald et al., 2016). The social memory test probes for the social recognition and memory of a conspecific and will be described further in Chapter 6. Under basal conditions, PNS females exhibit an impairment in social olfactory memory in comparison to control females (Grundwald et al., 2016). Acute stress exposure immediately prior to the social memory test, on the other hand, markedly improved the social memory performance in PNS females (no effect was observed in the male offspring). The social memory deficit was associated with lower vasopressin receptor type 1a (AVP-R1a; *Avpr1a*) mRNA expression in the anterior part of lateral septum and in the BNST (Grundwald et al., 2016). Improved social memory in the PNS females following acute stress is also accompanied by elevated AVP-R1a mRNA expression in both of these regions. Non-social olfactory memory, which involved recognition of spices, was not affected in either sex.

Together, these data demonstrate that performance of PNS offspring in different types of learning and memory tests is dependent on the brain region involved in performing the test, the sex of the animal, and can be further modulated by acute stress and chronic stressors.

Effects of PNS on reproductive traits:

Prenatal social stress also results in subtle differences in the reproductive axis of male offspring, resulting in longer anogenital distances and higher plasma follicular stimulating hormone (FSH) concentrations, with a trend towards increased plasma testosterone concentrations compared to controls (Ashworth et al., 2016). In PNS females, no alterations in the primary follicles or ovarian aromatase expression, and no differences in plasma progesterone were observed when compared to control female offspring (Ashworth et al., 2016, Brunton and Russell, 2010). These data suggest that the developing male reproductive axis is more sensitive to maternal stress than females, and increased FSH and testosterone may suggest that prenatal stress may enhance aspects of male reproductive development (Ashworth et al., 2016).

Prenatal stress also did not have any significant effect on maternal behaviour of the female PNS offspring when they became mothers, as the total time spent in behaviours such as pup retrieval, pup licking or grooming, arched back nursing were unchanged compared to controls (Grundwald, 2016).

Effects of PNS on glucose homeostasis and peripheral metabolism

Apart from the brain, offspring from socially stressed dams also show alterations in indices related to glucose metabolism and lipid homeostasis, which may indicate susceptibility to metabolic disease (Brunton et al., 2013). The effects are also sex-specific, where male PNS offspring exhibit increased glucose production in response to acute restraint stress; while females show poorer glucose tolerance and produce excessive levels of insulin following an oral glucose load (Brunton et al., 2013). Moreover, there is also a sex-dependent change in the expression pattern of genes involved in glucocorticoid and lipid metabolism (Brunton et al., 2013). Males show changes mostly in the liver and skeletal muscle, whereas in females the majority of changes are observed in subcutaneous fat, which may indicate differential risk for metabolic dysfunction between the sexes (Brunton, 2013).

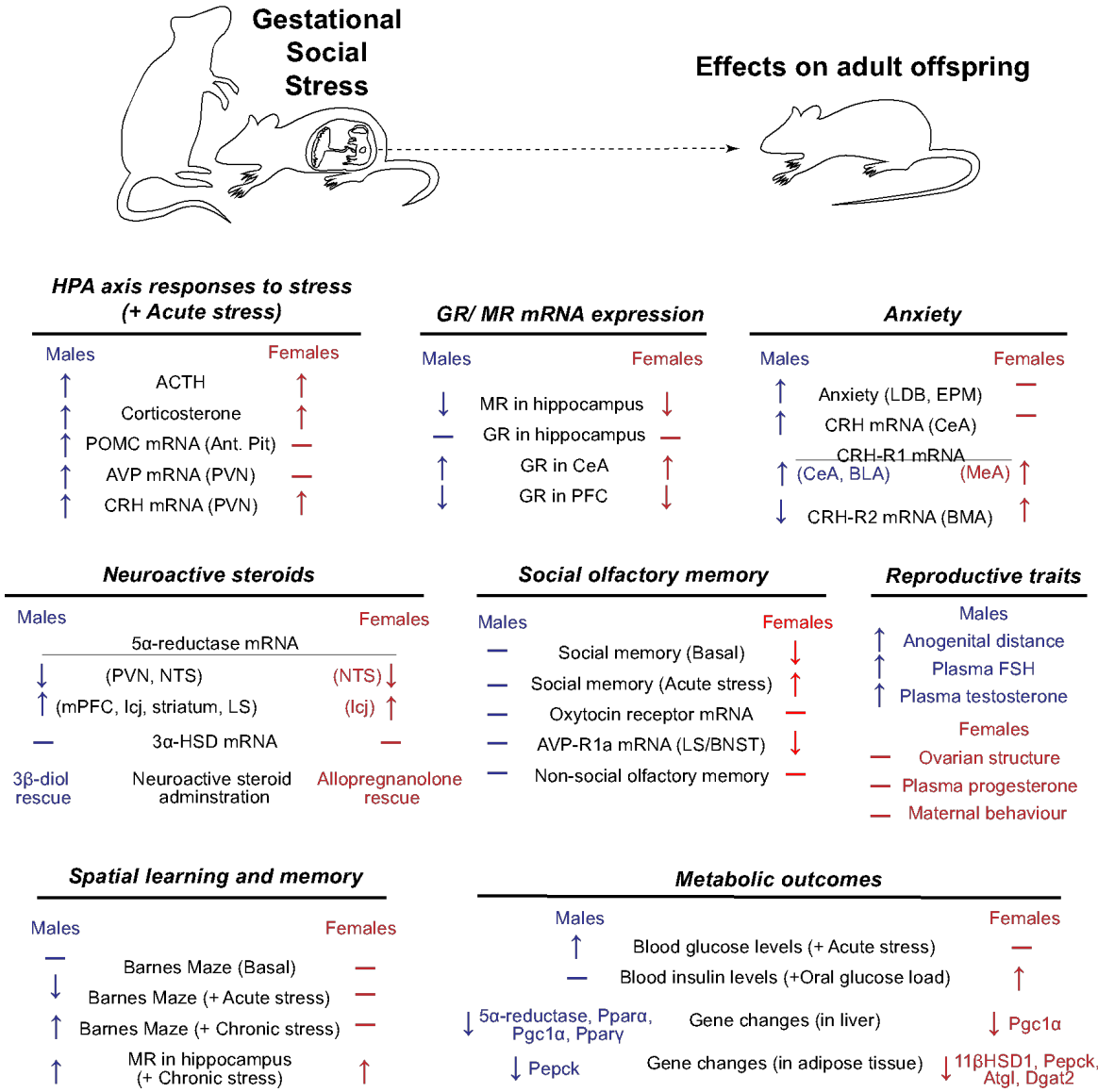


Figure 1.7: Summary of known behavioural and neurochemical characteristics of adult offspring for the prenatal social stress model. Summarised from the work of: Brunton and Russell, 2010, Brunton and Russell, 2015, Grundwald et al., 2016, Lai, 2016, Brunton et al., 2013, Ashworth et al., 2016. Blue: applies to males, in red: applies to females. ↑ denotes an increase in PNS, ↓ denotes a decrease in PNS, -- denotes no difference. Abbreviations: MR: mineralocortcoid receptor, GR: glucocorticoid receptor, POMC: pro-opiomelanocortin, PVN: paraventricular nucleus of the hypothalamus, LDB: light-dark box, EPM: elevated plus maze, CRH-R1 and R2: CRH receptor 1 and 2, PFC: prefrontal cortex, CeA: Central amygdala, MeA: Medial amygdala, BLA: Basolateral amygdala, NTS: nucleus of the solitary tract, lcj: the islands of Calleja, BNST: bed nucleus of the stria terminalis, LS: lateral septum, 3β-diol: 3β-androstanediol, AVP-R1a: vasopressin receptor type 1a, FSH: follicular stimulating hormone. Ppar: peroxisome proliferator-activated receptor, Pepck: phosphoenolpyruvate carboxykinase, Atgl: adipose triglyceride lipase, Dgat2: diglyceride acyltransferase 2, 11βHSD1: 11β-hydroxysteroid dehydrogenase type 1, Pgc1a: peroxisome proliferator-activated receptor gamma coactivator 1α

1.5.3 Mechanisms mediating "programmed" offspring outcomes in later life

A common theme to emerge in the outcomes of prenatally stressed offspring in later life is HPA axis dysregulation, which underlies many of the mood and affective orders described in the offspring (both human studies and animal models). Alterations in HPA axis functioning represents an allosteric state (section 1.2.2), which implies increased vulnerability to environmental challenges of any kind, increasing the risk of psychopathology, thus can also be considered an adverse outcome in itself (Huizink and de Rooij, 2018). The mechanisms underlying dysregulation of the HPA axis is multi-faceted (section 1.2.2) and some of the underlying processes pertinent to the prenatal social stress model have been covered in section 1.5.2. Here, other mechanisms that may underlie the aberrant phenotypes in the "programmed" offspring are introduced.

Imbalance of excitatory and inhibitory signals

HPA axis activation and inhibition are controlled by complex glutamate and GABA neurotransmitter pathways, which allows for the communication between different brain regions (section 1.2.1.2) (Herman, 2013). In rats, prenatal restraint stress increases glutamate transport in the hippocampus and reuptake in the frontal cortex of adult offspring (Adrover et al., 2015), and these rats also exhibit higher levels of NMDA receptors in frontal cortex, striatum and hippocampus compared to control rats (Berger et al., 2002). Moreover, subunit expression patterns of the NMDA receptor in the hippocampus are altered, which seem to contribute to anxiety-like behaviour in prenatally stressed rats (Sun et al., 2013, Kinnunen et al., 2003). Prenatal stress can alter inhibitory circuits, especially GABAergic circuits within and projecting to the hypothalamus, which are critical for regulating HPA axis reactivity (Fine et al., 2014). Subunit composition of $GABA_A$ receptors may also be altered in limbic regions following prenatal stress in rats and mice (Laloux et al., 2012, Jacobson-Pick et al., 2012). Whether or not some of these changes also occur in the prenatal social stress is not known and will be investigated in Chapter 6.

The role of neuroactive steroids

Altered excitatory or inhibitory neurotransmission may also be an indirect outcome of disrupted neurosteroidogenesis (section 1.3.6) (Brunton et al., 2015), which seems plausible given that the HPA axis hyperactivity and behavioural dysfunction associated with prenatal stress can be ameliorated by the administration of $GABA_A$-

modulating neuroactive steroids during adulthood in rat studies (Zimmerberg and Blaskey, 1998, Brunton et al., 2015). However, due to the difficulties associated with measuring steroids in brain tissue, only a few studies have attempted to directly quantify neuroactive steroid concentrations in the brain. Following the optimisation and development of a LC-MS method for the quantification of a panel of steroids in the brain, an investigation of whether neuroactive steroid production may be affected by prenatal stress, and if it underlies offspring behavioural phenotypes, is carried out in Chapter 4.

Neuroanatomical changes

In both rodent (Soares-Cunha et al., 2018, Mychasiuk et al., 2012) and human (Sarkar et al., 2014) studies, there is evidence for neuroanatomical changes especially in the limbic regions of the brain of PNS offspring, which may underlie aberrant behaviour (Lupien et al., 2009). Using a prenatal restraint stress model in the rat, a reduced number of granule cells in the hippocampus of adult female PNS offspring is observed, which could be a predisposing factor for the development of depression (Schmitz et al., 2002). There is also evidence of decreased neurogenesis in the rat hippocampus of offspring following prenatal stress, which could be linked to decreased learning and memory performance (Fatima et al., 2019, Lemaire et al., 2000). Dendritic morphology of neurones are also found to be altered in PNS rats, where there are reduced dendritic length and branching of neurones within hippocampal, prefrontal cortex and striatal regions, which can then affect neurotransmission (e.g. dopaminergic transmission in the nucleus accumbens of the striatum) (Mychasiuk et al., 2012, Martinez-Tellez et al., 2009).

It has been proposed that these changes arise from prenatal stress altering organisation of neuronal circuits of the neocortex and hippocampus during early brain development, thereby influencing the course of experience-dependent synaptic changes in later life (Mychasiuk et al., 2012). It is not known if neuroanatomical changes are altered in offspring of the social stress model, however some of these possible alterations will be investigated in Chapter 6.

Epigenetic mechanisms

Lastly, altered phenotypes in prenatally stressed offspring may involve epigenetic mechanisms (Meaney et al., 2007). Epigenetic “marks” are modifications on DNA,

such as DNA methylation and histone modifications, which provide a means through which gene expression can be altered (Kundakovic and Jaric, 2017, Zucchi et al., 2013). There is evidence that early prenatal stress in mice results in increased methylation of the GR promoter in the hippocampus of adult male offspring (Mueller and Bale, 2008). The methylation status of the GR promoter in cord blood mononuclear cells of 3 month old infants is also found to be increased if they were born to depressed mothers (Oberlander et al., 2008). Studies utilising synthetic glucocorticoids during guinea pig pregnancies have indicated that profound changes in DNA methylation, acetylation, and gene transcription begin in the foetal hippocampus (Crudo et al., 2013a), but specific patterns may change as the offspring develops (Crudo et al., 2013b). Therefore, maternal stress can be thought of as leaving an epigenetic ”mark” that is imprinted on the offspring genome during the foetal stages, which can either persist or be subsequently altered throughout the offspring’s life. These epigenetic marks thereby define the offspring’s behavioural phenotypes and also the vulnerability for diseases over their lifespan (Meaney et al., 2007, Cao-Lei et al., 2017)

1.5.4 Mechanisms mediating the transmission of stress signals from mother to foetus

A large majority of the research regarding the impact of chronic gestational stress has been focussed on the offspring outcomes (section 1.5.3). However, the mechanisms underlying the transmission of stress signals are also not fully understood. This section details some of the known mechanisms postulated to mediate the transmission of stress signals from the mother to foetus, reviewed in (Maccari et al., 2014, Boersma and Tamashiro, 2015, Brunton, 2013, Rakers et al., 2017). Mechanisms which usually involves all three components of the maternal-placental-foetal triad.

During stress in pregnancy, the mother plays an important role as the stress response is often maternally-initiated, especially in the case of psychological or social stress. Despite attenuation of the maternal stress axis during late pregnancy, as reviewed in rat studies in (Johnstone et al., 2000, Douglas et al., 2003, Brunton and Russell, 2008a) (section 1.4.1), the stress response is not completely abolished and stressful stimuli still results in the engagement of the maternal SNS and HPA axis and production of stress mediators (section 1.2.1). As the maternal and foetal blood systems do not mix, the maternal-foetal transfer of these mediators need to be facilitated by the placenta (Sadovsky and Jansson, 2015). These mediators can either cross the placenta directly, or lead to indirect changes in the physiology of the placenta (e.g. by affecting placental transport capacity or changing its endocrine function), which consequentially alters foetal physiology. Downstream effects of these mediators are also dependent on how the foetus interprets or modifies the signals, for instance through the metabolism of these mediators, or by controlling receptor expression for these mediators. Permanent changes may be induced in the foetus, or its physiology may be modified in a way that it increases its vulnerability to disease only in the event of a second hit in adulthood (Huizink et al., 2004).

Glucocorticoid programming:

Glucocorticoids, the end product of the HPA axis, are lipophilic and are often assumed to readily diffuse across the placental barrier into the foetal compartment (Cottrell and Seckl, 2009). Excessive glucocorticoid production from the maternal adrenal glands may therefore result in detrimental effects in the offspring, and this process is known by many as glucocorticoid programming, one of the key

mechanisms underpinning foetal programming (Barbazanges et al., 1996, Holmes et al., 2006, Seckl and Meaney, 2004, Cottrell and Seckl, 2009, Drake et al., 2007).

Two lines of evidence support this hypothesis, firstly, treatment with a synthetic glucocorticoid (e.g. dexamethasone) during pregnancy results in offspring having similar phenotypes to those prenatally stressed, observed from rat, human and guinea pig studies (Welberg and Seckl, 2001, Kapoor and Matthews, 2005), and secondly, maternal adrenalectomy, which prevents the production of maternal glucocorticoids, abrogates some of the effects of prenatal stress, observed in the rat model (Barbazanges et al., 1996).

However, these experiments do not provide a full mechanistic picture of how glucocorticoid programming occurs, as synthetic glucocorticoids do not truly mimic the action of maternal endogenous glucocorticoids like corticosterone and cortisol, as they are not a substrate for 11β-HSD2 and thus can cross the placenta freely, bypassing one of the natural "lines of defence" during pregnancy. Adrenalectomy also abrogates other compounds that may be secreted by the adrenal glands during stress (for instance, catecholamines from the medulla) and completely changes the HPA axis of pregnant dam, which may additionally confound the conclusions drawn from that experiment. Therefore, in as much as the effects of glucocorticoid programming is indisputable, how exactly maternal stress-induced programming occurs still remains much of an enigma in the field. Much of the focus has turned onto investigating the three endogenous lines of defence which serve to limit foetal overexposure to acute increases glucocorticoids (summarised in Fig 1.8), and whether they could be compromised in the event of chronic maternal stress.

There is evidence that chronic stress may affect the first line of defence, the maternal HPA axis in both rodents and in human populations, for instance, through disruption of negative feedback mechanisms, such that there is an elevated or prolonged production of plasma glucocorticoids (i.e. HPA axis is no longer hyporesponsive) (Takahashi et al., 1998, Duthie and Reynolds, 2013). Regardless, it is worth noting that even if maternal stress responses are dampened, responses are not completely abrogated and glucocorticoids are still produced whenever the stressful stimuli is present. As such, the integrity of the second line of defence, placental 11β-HSD2, may play an even more crucial role (Seckl et al., 1990, Seckl, 2017). Indeed, it has been found that increased levels of stress in pregnant mice

and rats results in significantly reduced activity and mRNA expression of placental 11β-HSD2, which could mean compromised ability to inactivate excess maternal corticosterone, allowing for increased corticosterone transfer (Welberg et al., 2005, Mairesse et al., 2007, Jensen Peña et al., 2012). In humans, maternal distress (measured by the Perceived Stress Scale) is associated with increased methylation of the *HSD11B2* gene in the placenta, which leads to downregulation of 11β-HSD2 mRNA and protein expression (Monk et al., 2016b). Lastly, there is also evidence that the rat foetus can produce glucocorticoids itself following stress (Ohkawa et al., 1991a), adding complexity to the mechanisms of glucocorticoid programming.

Therefore, in as much as glucocorticoids are recognised in the field as playing a role in the transmission of stress signals, gaps still remain, especially regarding how or the extent of which it occurs. Chapter 5 aims to further investigate whether some of these protective mechanisms are compromised in the gestational social stress model, thereby leading to aberrant offspring outcomes.

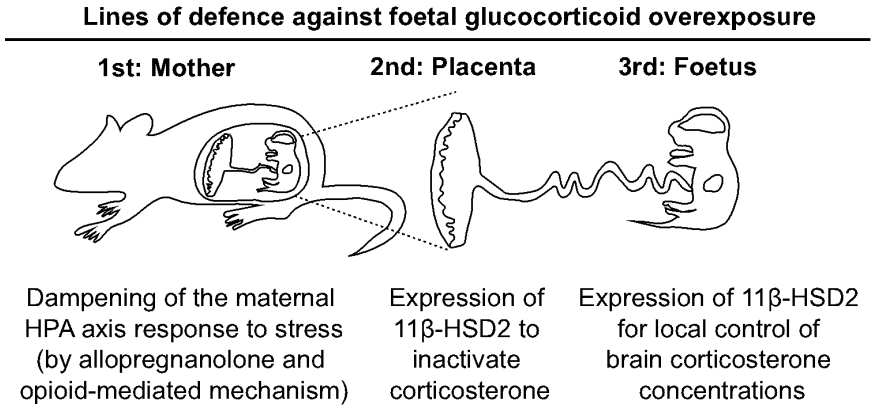


Figure 1.8: Three lines of endogenous defence against glucocorticoid overexposure. To recap, the first line of defence lies in the mother, where the HPA axis response to stress is dampened, and there is a reduced amount of ACTH and glucocorticoids produced following an acute stressor. Secondly, there is substantial expression of 11β-HSD2 in the placenta, which converts corticosterone/cortisol to their inactive 11-keto form, serving as the "glucocorticoid barrier" to control the maternal-foetal glucocorticoid transfer (Benediktsson et al., 1997). Lastly, in the foetus, 11β-HSD1 and 11β-HSD2 are expressed in the foetal tissues including the brain, and 11β-HSD2 serves as the third line of defence to further regulate local concentrations of glucocorticoids (Seckl, 1997, Chapman et al., 2013). Compromises in any of these lines of defence may result in foetal glucocorticoid exposure, leading to detrimental outcomes.

Catecholamines:

The sympathetic nervous system is also attenuated in pregnant rats, especially for adrenaline production following acute stimuli. Adrenaline production is decreased in pregnant as compared to non-pregnant women in response to heat stress (Vaha-Eskeli et al., 1992) and similarly, adrenaline responses are subdued in pregnant mice in response to air puff startle (Russell et al., 2008), although underlying mechanisms remain unknown. In both studies however, noradrenaline responses are not attenuated (Vaha-Eskeli et al., 1992, Brunton and Russell, 2015, Russell et al., 2008).

Noradrenaline is a potent vasoconstrictor which reduces blood flow via activation of α-adrenergic receptors present in the uterine and the placental vasculature (Rakers et al., 2017, Resch et al., 2003). There has been ample evidence in the sheep that an acute physical or psychological stressor can rapidly reduce uterine blood flow (Rakers et al., 2015, Dreiling et al., 2016). Chronic stress may further affect noradrenaline breakdown or reuptake, decreasing the capacity of the placenta to clear noradrenaline, and hence further reducing utero-placental perfusion (Piquer et al., 2017, Blakeley et al., 2013). This reduced utero-placental perfusion can potentially alter foetal metabolism by decreasing nutrient transfer and/or gaseous exchange, leading to foetal hypoxia or undernutrition (Gu et al., 1985, Gu and Jones, 1986). Consequently, this foetal hypoxia and nutrient restriction may then activate the foetus' own HPA axis, which in turn contribute to the foetal programming of HPA axis responsivity and anxiety-like behaviour in the adult offspring (Brunton, 2013, Nunez et al., 2008, Fan et al., 2009, Edwards and McMillen, 2002).

Oxidative stress:

Psychological stress is known to result in increased oxidative stress, both in humans and in rodents (Brunst et al., 2017, Picard and McEwen, 2018b, Irie et al., 2001). In humans, elevated markers of oxidative damage are observed with increased work stress in females (Irie et al., 2001); while in rodents, oxidative status is significantly increased in the brain, blood cells, heart, liver, and spleen following acute restraint stress (Spiers et al., 2016, Chen et al., 2014).

Although a moderate increase in maternal oxidative stress status is one of the physiological adaptations of pregnancy (Myatt and Cui, 2004, Burton and Jauniaux,

2011), constant or severe stress may tip this mild oxidative state over to one that causes adverse pregnancy outcomes. In clinical studies, increased oxidative stress status is correlated with lower birth weight and higher incidences of preterm delivery (Weber et al., 2014). This is also supported by the observation that maternal antioxidant administration in rats (e.g. hydrotyrosol and docosahexaenoic acid, DHA) can prevent deleterious offspring outcomes associated with gestational stress (Zheng et al., 2015, Feng et al., 2012).

In contrast to maternal stress hormones such as glucocorticoids, pro-oxidant molecules generally act as intracellular signalling molecules and do not have endocrine properties (Schieber and Chandel, 2014), and are unlikely to be directly transferred across the placenta due to their instability (Munro and Pamenter, 2019). Hence pro-oxidants probably act through an indirect mechanism via altering placental physiology (e.g. placental morphology, structure, function, or gene and protein expression patterns) (Sferruzzi-Perri and Camm, 2016). Antioxidants on the other hand, can be transferred to the foetal circulation (Kaempf-Rotzoll et al., 2003), but have similarly been found to exert some of their beneficial actions through altering placenta function in the sheep (Thakor et al., 2010) and rat (Richter et al., 2009). Oxidative stress as a potential mechanism of stress signal transmission in the prenatal stress model will be further explored in Chapter 6.

Immune system and cytokines:

It has also been proposed that the maternal immune system and the maternal release of cytokines may play a significant role in transferring psychological stress signals to the foetus. Cytokines are polypeptides that act as mediators of cellular signalling, modulating many processes involved in inflammation (Elenkov and Chrousos, 2002). Although there is a degree of immune suppression in human pregnancies in order to prevent rejection, the immune system is generally regarded to be modulated rather than attenuated (Mor and Cardenas, 2010), as there is still evidence that chronic psychosocial stress during pregnancy may result in an increase in maternal plasma pro-inflammatory cytokines (e.g. IL-6 and TNF-α) and poorer infant outcomes (Gustafsson et al., 2018, Coussons-Read et al., 2007). As for the maternal-foetal transfer mechanism, some cytokines (e.g. IL-6) have been found to be able to cross the placental barrier from the maternal to foetal circulation in the rat (Dahlgren et al., 2006). In mice, maternal immune activation can also

trigger placental production of IL-6 and release directly into the foetal circulation (Hsiao and Patterson, 2011). Cytokines are extremely important for neurogenesis and also the development of glial cells during foetal brain development (Deverman and Patterson, 2009). Alteration in levels of cytokines in the foetal compartment therefore can disrupt normal brain development in rat and human foetuses (Ratnayake et al., 2013). Cytokines can also interact with the stress response system by further stimulating the HPA axis and promoting glucocorticoid release, thereby contributing to glucocorticoid programming (Silverman and Sternberg, 2012).

Serotonin (5-HT) system

It has been suggested that psychosocial stress by crowding during rat pregnancy increases maternal plasma tryptophan (precursor of serotonin) concentrations, together with elevated foetal brain levels of tryptophan, serotonin, and 5-hydroxyindoleacetic acid (5-HIAA; breakdown product of serotonin) (Peters, 1990). Given that serotonin is crucial for normal placentation and normal brain development during embryonic, foetal as well as postnatal stages, in both rat and human studies, the stress-induced increase of serotonin and 5-HT metabolites in the foetal brain may therefore interfere with developmental processes (Bonnin and Levitt, 2011).

Neuroactive steroids

Neuroactive steroids are modulatory in nature, which imply that they can further modify any of the mechanisms mentioned above. In the foetus, increases in allopregnanolone production following acute stress, such as hypoxia, and has been proposed to represent an endogenous neuroprotective mechanism in the foetal sheep to acute stressors (Nguyen et al., 2004). It is possible that chronic stress-induced changes in the mother could impair this endogenous protective mechanism in the foetal brain, especially when it has been shown that synthetic corticosteroid administration to the guinea pig foetus suppresses expression of the enzymes involved in neurosteroidogenesis (McKendry et al., 2010). Allopregnanolone treatment in the mother during late gestation was also able to reduce the deleterious effects of prenatal stress on the offspring in rats, again suggesting that reduced allopregnanolone levels during pregnancy could be an underlying factor contributing to adverse developmental outcomes (Zimmerberg and Blaskey, 1998).

Maternal behaviour of stressed dams

Lastly, prenatal stress may also lead to changes in the maternal brain that can affect post-partum maternal care (Champagne and Meaney, 2006). Although the post-partum period is not a key focus of this thesis, maternal care is known to affect the growth and development of the neonate's HPA axis postnatally.

The period of HPA axis hyporesponsiveness in the rat neonate is maintained by the dam's behavioural interaction with the pups, as 24 hr separation from the mother results in the deprived neonate having elevated basal levels of corticosterone, and exhibiting a robust corticosterone and ACTH response to stressors (Levine, 2002). A lower frequency of licking and grooming of the pups by the dam also leads to a more anxious phenotype in the offspring during adulthood, coupled with a hyperactive HPA axis (Liu et al., 1997). Altered maternal-pup interactions can therefore be a mechanism that can affect postnatal brain development, and similar to *in utero* stress, can consequently produce long-lasting alterations that extends into adulthood, again fitting into the DOHaD framework (Kalinichev et al., 2002, Wigger and Neumann, 1999). In children, early neglect can impact the development of brain circuitry that regulate stress and emotional function, increasing the likelihood of psychopathology (Bremner and Vermetten, 2001, Gunnar and Quevedo, 2007).

For the mother, the transition between pregnancy to the post-partum period also requires significant physiological and behavioural changes, and gestational stressors could alter these adaptive processes, leading to poorer maternal care. For instance, stress during pregnancy is known to decrease the expression of licking and grooming behaviour in rats and mice, which can in turn affect development of the neonatal HPA axis (Champagne and Meaney, 2006). Moreover, gestational stress can result in sustained production of elevated levels of corticosterone during the postnatal period in rat dams, which may further affect maternal behaviour (Pfister and Muir, 1989). These lines of evidence provide an alternative view that gestational stress can indirectly lead to programming effects in the offspring not only *in utero*, but indirectly via changes in postnatal maternal behaviour and maternal care. Nonetheless, unpubished observations from the Brunton lab indicate no significant differences in maternal care following exposure to social stress during pregnancy, suggesting this does not explain the altered phenotypes observed in the offspring.

1.6 Specific aims and hypothesis:

Thus far, the introduction has identified some gaps that are present in the field of foetal programming and the developmental origins of health and disease (DOHaD), and briefly pointed out in each section how this work aims to address them. The general aims of this work can be classified into three main themes, and is summarised in Fig 1.9, with the specific aims elaborated below.

Aim 1: To develop a liquid chromatography-mass spectrometry (LC-MS) method to quantify a panel of steroids, especially those that are involved in modulating stress responses. These include corticosterone, the final output of the HPA axis, and its precursors (e.g. DOC), and inactive metabolite 11-DHC. Other important steroids considered include the $GABA_A$ positive modulators allopregnanolone and THDOC due to their anxiolytic nature and their role in attenuation of the stress response, and their respective precursors (e.g. DHP and progesterone; DHDOC and DOC). The method also aimed to include several sex steroids (e.g. progesterone, oestradiol, and testosterone and its metabolites DHT, 3α-androstanediol and 3β-androstanediol) which may be informative in accounting for the sex differences in the response to stress (Chapter 3).

Aim 2: To investigate the factors mediating prenatal "programming" of the offspring, i.e. the mechanisms that are transmitting the stress signals from the mother to foetus, at GD20. Specific questions include:

1. Are glucocorticoids involved in the transmission of maternal stress to the foetuses? (Chapter 5)
2. Are there altered steroid levels (in the placenta and foetal tissues) following social stress? (Chapter 5)
3. Is there a role for oxidative stress in mediating the transmission of stress signals during pregnancy? (Chapter 6)

Aim 3: To investigate the mechanisms underlying the expression of prenatally "programmed" phenotypes in the adult offspring. Specific questions include:

1. Do PNS offspring exhibit HPA axis hyperactivity in response to acute swim stress? (Chapter 4)

2. Are there differences in neuroactive steroid production between control and PNS offspring, under basal conditions and in response to acute swim stress? (Chapter 4)
3. Do PNS offspring express depressive-like phenotypes? (Chapter 6)
4. Are there other morphological and neurochemical changes in the brain of PNS offspring that may underlie altered behaviour? (Chapter 6)

This work first begins with the development of an LC-MS technique to quantify steroids in the brain (Chapter 3), which allows for the testing of several hypotheses related to aims 2 and 3.

It is first hypothesised that changes in concentrations of neuroactive steroids in the brain and periphery of adult PNS offspring is a mechanism that underlies their aberrant behaviours and physiology, such as HPA axis dysregulation (Aim 3; Chapter 4). Secondly, focussing on events occurring during the pregnancy, it is hypothesised that prenatal stress leads to a compromise in the protective functions of the placenta (e.g. decreased 11β-HSD2 expression), and subsequently increased glucocorticoid exposure in the foetus (Chapter 5). Nonetheless, as the mechanisms regulating the transmission of stress from the mother to foetus is complex, it is also hypothesised that other mechanisms such as a decrease in neuroactive steroid concentrations in foetal tissues (also Chapter 5), or an increased oxidative stress status in the placenta (Chapter 6) can contribute. Finally, it is also hypothesised that interventions during pregnancy, such as maternal antioxidant treatment (Chapter 6), may be a viable method to prevent detrimental outcomes associated with prenatal stress in the offspring.

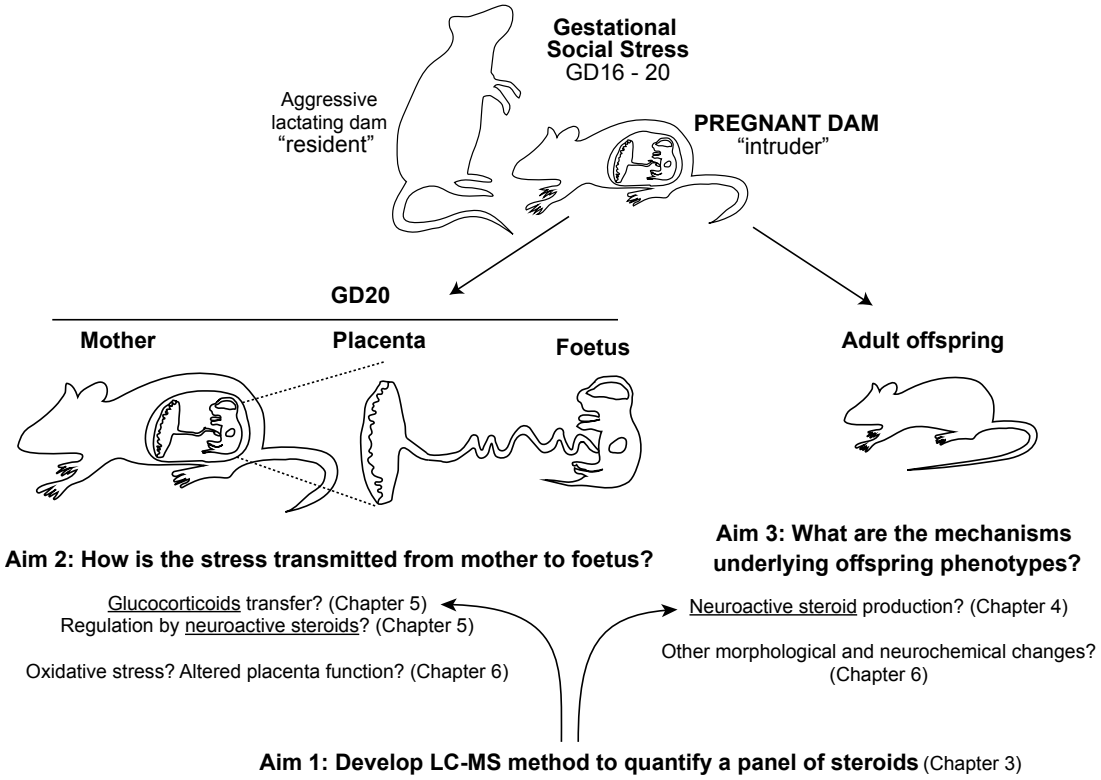


Fig 1.9: Summary of thesis aims.

Chapter 2: General Materials and Methods

This chapter first provides a list of all the *in vivo*, *in vitro*, molecular and chemical techniques used to achieve the three aims mentioned in the introductory chapter (Table 2.1). It then elaborates on some of the common techniques that are utilised repeatedly in this work (e.g. animals used, stress paradigm used, tissue collection, in situ hybridisation, LC-MS), whilst techniques that are unique to each chapter will be detailed in the relevant chapters. Although a finalised LC-MS method for steroid quantification is included in this chapter, the optimisation and validation of the method, which forms a substantial part of the thesis, will be presented in Chapter 3.

Chapter	Summary of the techniques used
3	1. Liquid chromatography-mass spectrometry (LC-MS) quantification of steroids a. Optimisation of the method b. Validation of the method c. Application of the method
4	1. Prenatal social stress 2. Acute swim stress (adult offspring) 3. Conscious decapitation and tissue collection 4. Dissection of brain regions 5. LC-MS quantification of steroids
5	1. Prenatal social stress 2. Conscious decapitation and tissue collection 3. LC-MS quantification of steroids 4. *In situ* hybridisation a. Placenta: 11β-HSD2, GR b. Foetal brain: 11β-HSD1, 11β-HSD2 5. Western blotting (placenta, 11β-HSD2)
6	1. Prenatal social stress 2. Intravenous administration of antioxidants to pregnant rat 3. Behavioural tests a. Anxiety (light-dark box and elevated plus maze) b. Depression (Forced swim test and sucrose preference test) c. Social memory test 4. Assessment of HPA axis dysregulation a. Surgery b. Acute restraint stress c. Blood collection 5. Radioimmunoassay (plasma corticosterone and ACTH) 6. *In situ* hybridisation

	a. Placenta: 11β-HSD2 b. Adult and juvenile brain: CRH 7. CO_2 overdose and tissue collection 8. Conscious decapitation and tissue collection 9. Oxidative stress assay 10. *Ex vivo* placental culture and *in vitro* neuronal culture 11. LC-MS quantification of steroids

Table 2.1 Overview of all techniques used in this thesis. Techniques that are common to all chapters are underlined and are detailed in this chapter.

2.1 Animals

All animal experiments were carried out in the Roslin Institute Biological Resource Facility, approved by the Institute's Animal Welfare and Ethical Review Body and performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and the European Directive (2010/63/EU). Sprague-Dawley (SD) rats were used for all experiments.

Female SD rats were purchased from Charles River (Margate, Kent, UK) and were acclimatised to the facility for at least a week before any procedures. Rats were group-housed (4 females) in individually ventilated cages (IVCs). Female rats that were intended to be used as aggressive lactating dams (i.e. to induce social stress) arrived in the facility one week before the experimental batch, were heavier (250 – 300 g) as compared to the experimental dams (200 – 225 g). Rats were maintained on a 12:12 hr light-dark cycle (lights on at 07:00 hr), under controlled temperature (22^{o}C) and humidity (55%). Rats were provided with food and water *ad libitum*, and pregnant and lactating dams were given a 50:50 mixture of 14% and 19% protein diet (Harlan Teklad).

For mating, one female was housed in a breeding cage with a wire mesh with one sexually experienced male (either bred within the facility or also purchased from Charles River). Mating was confirmed by the presence of a semen plug in the breeding cage the following morning, and this was designated as gestational day (GD) 1. Females were returned to same sex group-housing until GD16 (for experimental dams) where they were transferred to single IVCs. For lactating aggressive "residents", they were group housed until GD21, where they were transferred to individual open top cages in a separate room in the animal facility.

Following parturition, both dam and pups were left largely undisturbed except for routine husbandry. Pups were not cross-fostered, and were checked for litter size and weights at postnatal day (PND) 0, PND 8 and PND 23. Pups were weaned and separated by sex at PND 23 into IVCs. Depending on the litter size, sex ratio, nature of the experiments, as well the age and weights of the rats, the number of animals in each cage at any one time (from after weaning through to adulthood) varied from 4 to 10. Rats were never individually housed unless otherwise stated.

Three cohorts of pregnant rats were generated for the purposes of the thesis, the details of which are briefly outlined below. Detailed experimental design for each cohort of rats will be elaborated in the corresponding chapters.

Cohort	Pregnant rats	Offspring	Corresponding chapters
1	Pregnant rats were exposed to 5 days of gestational stress from GD16-20 and gave birth to offspring. n=10 each group, two groups in total	Effects of gestational stress on offspring neuroactive steroid concentrations were investigated in 7 week old offspring	Chapter 4
2	Pregnant dams were administered antioxidant or saline at GD16, exposed to 5 days of gestational stress (GD16-20), and gave birth to offspring. n=8 each group, four groups in total	Effects of gestational stress and maternal antioxidant administration on offspring behaviour and physiology were investigated in PND30 and 9 week old offspring	Chapter 6
3	Pregnant dams were administered antioxidant or saline at GD16, underwent 5 days of gestational stress, and were culled at GD20. Effects of gestational stress on the physiology of pregnant dams were investigated. n=7 each group, four groups in total	Foetuses culled before birth on GD20. Effects of gestational stress on foetal physiology was investigated on GD20.	Chapter 5 and Chapter 6

Table 2.2: Overview of three cohorts of pregnant rats used for this thesis

2.2 Prenatal stress/ Resident intruder paradigm

A modified resident-intruder paradigm was carried out as described in (Brunton and Russell, 2010). Aggressive lactating dams (i.e. “residents”) were generated by mating them one week ahead of the experimental dams. Lactating dams were housed individually in open-top cages two days before parturition and throughout lactation, in a separate room from experimental pregnant rats. Experimental pregnant females (“intruders”) were transferred to the home-cage of the “residents”, for 10 min each day from GD16 to GD20, between 10:00 – 14:00. Experimental pregnant dams (“intruders”) were always paired with an unfamiliar lactating “resident” each time to prevent habituation. Behaviours were observed and manually scored (where biting – 1 point, biting and pinning down – 2 points, biting and tumbling over – 3 points) to monitor severity of the encounters. Latency to the first attack was also recorded. Experimental pregnant dams were returned to their IVCs immediately after the 10 min social stress, except for experiments where tissues were collected on GD20, where pregnant dams were immediately killed after the 10 min social stress bout.

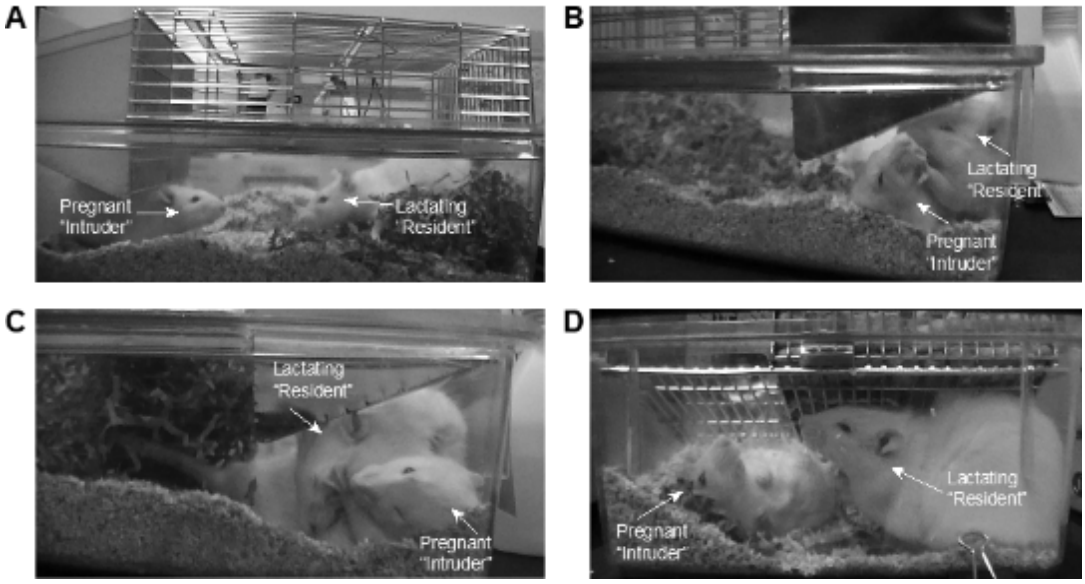


Figure 2.1: Resident intruder paradigm and examples of attacks. A) Pregnant ‘intruder’ introduced into the home cage of the aggressive lactating ‘resident’. B) Lactating ‘resident’ pinning down and biting the face of the pregnant ‘intruder’. C) Lactating resident pinning down and biting torso of pregnant ‘intruder’. D) Pregnant intruder assuming a submissive position following a bout of attack.

2.3 Killing and tissue collection

At the end of procedures, killing was carried out either by conscious decapitation or CO_2 overdose for tissue collection for ISH and LC-MS. As CO_2 inhalation and other forms of inhalation anaesthesia are known to act as acute stressors that alter the concentrations of neuroactive steroid in brains of rats (Barbaccia et al., 1994, Barbaccia et al., 1996, Taves et al., 2011), conscious decapitation was carried out if neuroactive steroid concentrations needed to be determined. In both cases, killing was carried out in a "post-mortem" room separated from the experimental or housing rooms, and rats were rapidly transferred from housing/experimental rooms to the "post-mortem" room using a transport container. Killing was carried out between 10:00 – 14:00 each day.

For conscious decapitation, the killing of the rats was carried out swiftly by placing the rats in a pliable plastic cone and were decapitated with a guillotine immediately. The loss of consciousness following decapitation occurs rapidly (within seconds) and is humane (van Rijn et al., 2011). Trunk blood was collected from rats following conscious decapitation, into chilled collection tubes containing 0.5 ml of 0.5% (w/v) EDTA kept on ice. For CO_2 overdose, rats were placed in a chamber with a rising concentration of CO_2 until respiration ceases (process takes approximately 3 min) and were decapitated to confirm death and to remove the brain.

In all cases, the brain was rapidly and carefully removed from the skull. The process involves chipping away the bone using rongeurs, starting at the back of the skull (the foramen magnum), over the cerebellum and forward towards the eyes (Paul et al., 2008). Once the brain is exposed, the meninges were carefully removed, olfactory bulb is severed and the cerebellum and brainstem were separated from the rest of the brain by cutting along the transverse fissure. The brain is gently pried away from the base of the skull with a spatula, the optic and trigeminal nerves severed with scissors and transferred onto a piece of foil for freezing on dry ice, or directly into 15% sucrose in 4% paraformaldehyde in the case of fixed brains. Killing with perfusion is described in Chapter 6.

In Chapters 5 and 6, where pregnant rats were decapitated, placenta and foetuses were also collected. A ventral midline incision was made on the torso of the decapitated pregnant dam, through the subcutaneous layer and peritoneum, and the uterus was pulled out of the abdominal cavity. Foetuses were rapidly removed from

the amniotic sacs, detached from the placenta by cutting of the umbilical cord. Upon determination of sex (on the basis of anogenital distance), foetuses were decapitated using scissors and trunk blood was collected using EDTA-coated capillary collection tubes (Microvette CB 300 µL, Sarstedt, Germany). Foetal trunk blood were collected from all foetuses and were pooled by sex within a litter due to the small volumes of blood that could be obtained, and were kept on ice once collected. Foetal brains were carefully removed from the skull using scissors and a spatula and were laid out on foil. The placentae were gently dabbed on clean tissue to remove excess blood and the maternal decidua was removed. Samples of maternal and foetal liver were also collected in 1.5mL Eppendorf tubes.

Brains, placenta and liver were frozen on dry ice or in liquid N_2, and stored at -80^{o}C until further use. At least three placenta and foetal brains per sex were collected for each litter (total of 7-8 litters per treatment group). One male placenta, one female placenta, one male brain, one female brain per litter were then picked at random for in situ hybridisation, while another similar set consisting one of each were picked at random for homogenisation for LC-MS. Both maternal and foetal trunk blood were centrifuged at 1500 g for 20 min at 4^{o}C, and the plasma was separated, aliquoted into fresh tubes and stored at either -80^{o}C or -20^{o}C until further use in radioimmunoassays or LC-MS analysis.

2.4 *In situ* hybridisation

In situ hybridisation (ISH) was carried out to investigate mRNA transcript expression in tissue sections. Radioactive ISH used in this thesis is based on the principle of complementary binding of a radiolabelled nucleic acid probe to specific target sequences of DNA or RNA in fixed tissue, followed by visualisation of the radioactive signal. Single-stranded RNA probes were used in this study. The advantage of ISH is that it provides information about the mRNA expression of single cells in its true anatomical location, which is especially helpful given the cellular heterogeneity present in the brain (Carter et al., 2010). The hybridisation signal in the form of silver grains is also fairly permanent, and can be visualised on bright-field light microscopy without the need for confocal microscopy.

The ISH process carried out in this thesis using riboprobes incorporating ^{35}S-labeled UTP has been carried out routinely in the laboratory and was first published in (Brunton et al., 2009). An overview of the ISH process, which requires generation of probes, hybridisation, and the detection and analysis of signal is provided in Fig 2.2. Briefly, following propagation, plasmids containing cDNA of the gene of interest were linearised and purified. Using this linearised template, radioactive probes were then synthesised via RNA transcription, where ^{35}S–UTP were incorporated into probes. Radioactive probes were then hybridised with tissue sections on slides (section 2.4.3), which have been cut, fixed and pre-treated beforehand (section 2.4.1). Hybridised probes were then exposed to radiosensitive emulsion before development and counterstaining (section 2.4.4).

Prior to the ISH experiment, all stainless steel equipment, slide racks, and glassware were heat-sterilised in a 200^{o}C oven and all surfaces were sprayed with an RNAse Surface Decontaminant (RNAse Away, ThermoFisher, UK) to reduce RNAse contamination. Diethylpyrocarbonate (DEPC; from Sigma UK) treated ultrapure water (ddH_2O) and 0.1M PBS were autoclaved before use. All steps were performed at room temperature (RT) unless otherwise stated.

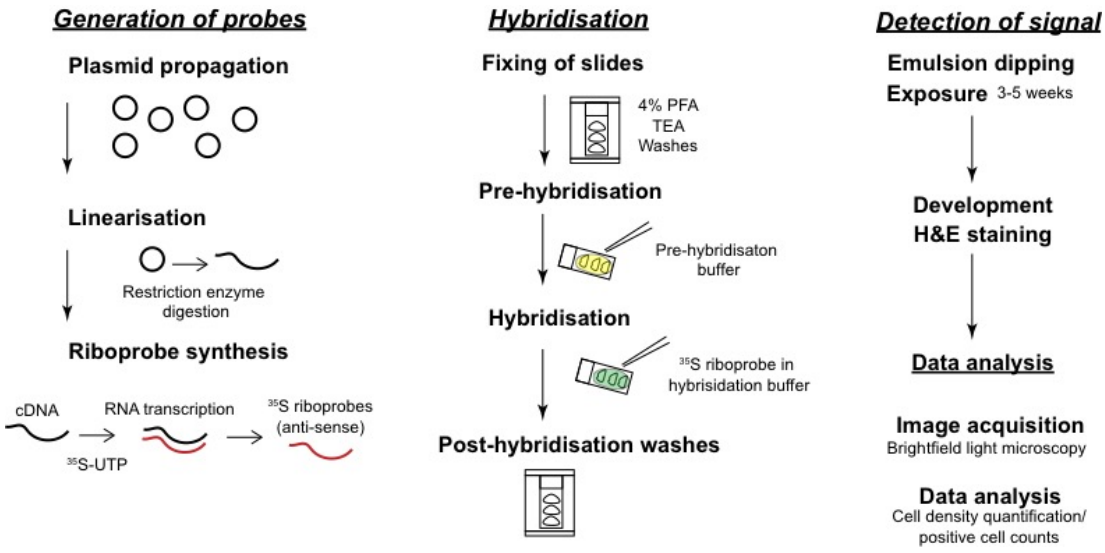


Figure 2.2: Schematic showing a summary of the ISH process.

2.4.1 Tissue sections

Frozen tissue was mounted (but not embedded) on OCT (Tissue-Tek) and 16µm sections were cut using a cryostat at -19°C (Leica CM1850), then thaw-mounted on Polysine adhesion slides (ThermoScientific). Rat brains were cut coronally, and relevant regions in the adult and juvenile rat (i.e. the amygdala) were identified with the help of marker sections (collected every 16th section) stained with toluidine blue, referenced to a stereotaxic brain atlas (Paxinos and Watson, 2007). Foetal brains were cut at the level of the hippocampus with the help of an atlas for the developing rat brain (Paxinos et al., 1994). Placenta were cut transversely, so that the junctional zone and labyrinth layer were represented in each section. Marker sections were collected on gelatine subbed slides (1% gelatine with chromium), and fixed with acetic alcohol fixative (4% w/v formaldehyde and 5% v/v acetic acid in ethanol) and stained with 1% toluidine blue for visualisation. Tissue sections on slides were stored in -80°C until processing for ISH.

2.4.2 Generation of riboprobes

Plasmid propagation: Plasmids containing an ampicillin-resistance gene and target DNA sequences inserted into the multiple cloning region were obtained from various sources, detailed in the relevant chapters. Plasmids were transformed and propagated using HB101 competent cells (Promega, UK). Ampicillin-resistant

colonies were cultured overnight, and then purified using the Qiagen HiSpeed plasmid midi kit as per manufacturer's instructions. DNA concentration of the purified plasmids were then quantified using a NanoDrop ND-1000 spectrophotometer, after which plasmid DNA was aliquoted and stored at -20^{o}C. Propagation of the plasmids were carried out by Mrs Helen Cameron (for CRH and GR) and Ms Joana Fernandes (for 11β-HSD1 and 11β-HSD2).

Linearisation of plasmid DNA: Plasmid cDNA was linearised using restriction enzymes. Restriction enzymes for each of the different probes are detailed in the relevant chapters. Additionally, plasmid cDNA were incubated with different restriction enzymes to generate antisense or sense probes. Antisense probes, which are complementary to and therefore will hybridise to mRNA transcripts of interest, are the probes used in the quantification of target mRNA expression. Sense probe sequences, on the other hand, are identical to the mRNA sequence of interest and will not bind, and were used as negative controls. Additionally, a double digest reaction was also set up, where both restriction enzymes were incubated with the DNA as a diagnostic method to verify insert size.

Purification of linearised DNA: Linearised cDNA (for both sense and antisense digests) were purified using phenol chloroform extraction, where equal volumes of phenol/chloroform/isoamyl alcohol (Sigma, UK) was first added to the solution containing linearised plasmid and the mixture was vortexed and centrifuged (13000 rpm, 5 min). The top layer was removed and then extracted again by the addition of an equal volume of chloroform/isoamyl alcohol (Sigma, UK), and were vortexed and centrifuged as above. The top layer was collected again and precipitated with 2 volumes of ethanol and 1/10th volume of 0.5 M NaCl on dry ice for 10 min. After centrifugation, the pellet was air-dried and resuspended in 15 µL of DEPC-treated ddH_2O. Purified linearised cDNA (for both antisense and sense probes), and the double digests were separated by agarose gel electrophoresis, to check for the insert size, integrity of the plasmid cDNA and to ensure complete linearisation, without contaminants or supercoiled DNA. Linearised cDNA were then quantified using the NanoDrop before being RNA transcribed.

Synthesis of ^{35}S riboprobes via RNA transcription: Riboprobes were generated by transcription of the linearised cDNA, using RNA polymerases which initiates RNA

synthesis at the specific promoters. Transcription was carried out using the Promega Riboprobes System kit as per manufacturer's instructions. Linearised cDNA template (2 ng in 2 µL) was added to solution containing 4 µL of 5X transcription-optimised buffer, 4 µL mixed bases (containing 2.5 mM each of ATP, GTP and CTP), 5 µL of ^{35}S-UTP (Perkin-Elmer #NEG039, 0.46 MBq/µL), 2 µL of 100mM DTT, 1 µL of RNAse inhibitor (2500U/ml, RNasin, Promega), 2 µL of appropriate RNA polymerase and incubated for 2 hrs. Following transcription, 2 µL DNAse was added and incubated at 37^{o}C for 15 mins, to degrade the cDNA template. To remove unincorporated nucleotides, the solution underwent size-exclusion chromatography using Illustra NICK Columns (GE Healthcare Life Sciences, Little Chalfont, UK). Purified ^{35}S-UTP labelled riboprobes were eluted in 400 µL TE buffer solution (1M Tris, 500 mM EDTA at pH 7.4). 1 µL of the eluted radioactive riboprobe was aliquoted into 3.5ml scintillation fluid (Optiphase HiSafe 3, Perkin Elmer) and the radioactivity (in counts per minute; cpm) was measured in a β-scintillation counter. Radioactive riboprobes (both sense and antisense) were stored at -20^{o}C until further use.

2.4.3 Hybridisation

Tissue fixation: Slides stored in -80^{o}C were pre-selected on dry ice depending on the region of interest, with the help of marker sections. 4% (w/v) paraformaldehyde (PFA) was prepared a day earlier by dissolving PFA (Sigma) in DEPC-treated 0.1M PBS with NaOH pellets, pH adjusted to 7.2-7.4 at RT and chilled overnight at 4^{o}C. Sections were first fixed in 4% PFA (in DEPC-treated 0.1M PBS, pH 7.2-7.4) for 10 min, then washed twice in DEPC-treated 0.1M PBS, all carried out using slide racks. Sections were then acetylated in TEA (consisting of 1.5% (v/v) triethanolamine, 0.25% (v/v) acetic anhydride, pH 3 in ddH_2O) for 5 min, then washed twice for 3 min in DEPC-treated 0.1M PBS. Sections then underwent dehydration with alcohol of increasing concentrations prepared in DEPC-treated ddH_2O (50%, 70% and 90%) for 2 min each, and then were air-dried before pre-hybridisation.

Prehybridisation: Pre-hybridisation saturates tissues with buffer before a probe is introduced, decreasing the risk of unspecific binding. Slides were pre-hybridised with 200 µL of 1X prehybridisation buffer in a humidified chamber at 50^{o}C. Prehybridisation buffer was prepared and stored as a 2X solution in -20^{o}C and were thawed and diluted 1:1 with deionised formamide to form 1X prehybridisation buffer (Severn Biotech, UK) on the day of use. 2X prehybridisation buffer consisted of a

final concentration of 1.2 M NaCl, 20 mM Tris pH 7.6, 2x Denhardt's Solution, 2 mM EDTA, 1mg/ml salmon testes DNA, 0.25 mg/ml yeast tRNA in DEPC-treated ddH_2O (all from Sigma, UK).

Hybridisation: After 2 hr of pre-hybridisation, 1X prehybridisation buffer was drained off and the sections were flooded with 200 µL of hybridisation solution containing the radiolabelled probe. To prepare the hybridisation solution, probes were added to 1X hybridisation buffer, heated for 10 min at 70^{o}C and then briefly cooled on ice before adding 0.015% (v/v) 1M DTT. The volume of radiolabelled probe required (from section 2.4.2) was calculated based on radioactivity measurements obtained in the β-scintillation counter. The hybridisation solution has a final radioactivity of 2.6 x 10^6 cpm per 200 µL of solution. Slides were flooded with 200 µL of hybridisation solution and incubated in a humidified chamber at 55^{o}C overnight (ca. 18 hr). Hybridisation buffer was prepared and stored as a 2X solution, diluted 1:1 with deionised formamide (Severn Biotech, UK) into 1X hybridisation buffer freshly before the addition of the radiolabelled probe. 2X hybridisation buffer consisted of 1.2 M NaCl, 20 mM Tris pH 7.6, 2x Denhardt's Solution (Sigma), 2 mM EDTA, 0.2 mg/ml salmon testes DNA, 0.25 mg/ml yeast tRNA, 200 mg/ml dextran sulphate in DEPC treated ddH_2O (all from Sigma, UK).

Post-hybridisation RNAse treatment and washes: After overnight hybridisation (18 hr), the hybridisation solution was drained off and the slides were rinsed in 2X saline sodium citrate (SSC), first individually three times, then in a slide rack for another three times. 2X SSC consisted of 0.3M sodium chloride and 0.03M sodium citrate in ddH_2O. 200 µL of RNAse A solution was added to slides, incubated in a humidified chamber for 60 mins in 37^{o}C to digest any unbound probes. RNAse A solution is prepared using 0.05% (v/v) RNAse A (30-33mg/ml, Sigma) in 0.5 M NaCl, 10 mM Tris (pH 7.6) and 1 mM EDTA. Following 60 min of incubation, RNAse A were drained off and slides were first rinsed in 2X SSC, once individually, and one additional time using a slide rack. Washes were then carried out, where they were first incubated in 2x SSC for 30 min RT, followed by more stringent "hot" washes, where sections were incubated in 0.1X SSC in a 60^{o}C water bath for 50 min three times. After the final wash, slides were dehydrated with increasing amounts of alcohol (2 mins each in 50%, 70% and 95% of ethanol in 0.3M ammonium acetate) before being air-dried.

2.4.4 Detection of radioactive signal

Emulsion dipping and exposure: Slides were then dipped in molten auto-radiographic emulsion (Ilford K5 nuclear emulsion, diluted to 70% with ddH_2O). Emulsion dipping was carried out in a warm water bath (42^{o}C) under safelight conditions. Slides were left in a light-proof lead box at RT until dry, then packed into slide boxes with desiccants and left to expose in the dark in 4^{o}C. Exposure times ranged from 3.5 weeks to 5 weeks, depending on probe.

Development and staining: Sections were incubated in developer solution (Phenisol Developer, Ilford; 6 min incubation) and rinsed in ddH_2O before being fixed (Hypam Rapid Fixer, Ilford; 2 x 6 min incubation). Following two washes with ddH_2O (5 mins each), sections underwent haematoxylin & eosin (H&E) counterstaining and dehydration on the Leica Autostainer XL, to enable visualisation of cell nuclei. Slides were incubated with haematoxylin (30 sec), washed, followed by Scot's tap water substitute (2 min), washed, and lastly with eosin (2 min). After washing, slides were then dehydrated through a series of alcohols of increasing concentration (70%, 95% and 99%), followed by alcohol/xylene (1:1 v/v) and finally xylene. Slides were immediately cover-slipped with DPX mounting media (Sigma, UK) and left to dry overnight.

2.4.5 Image acquisition and data analysis

Silver grains were visualised with the Nikon Ni1 brightfield microscope and acquired as TIFF images using the ZEN 2 blue software (Carl-Zeiss, Oberkochen, Germany). Hybridisation was quantified by either positive cell counts or grain density.

Positive cell counts analysis was carried out at 10X or 20X magnification, and cells with grain density >5 times above the background were considered positive. Background refers to non-specific hybridisation signal, and was defined as the grain density in adjacent brain regions where the gene is not known to be expressed.

Grain density calculation were carried out in images captured at 20X magnification. For the digitisation of the images, white balance was set at automatic for each image and exposure was kept consistent across images. Images were saved in TIFF format, and converted into 16-bit using Fiji software (ImageJ, NIH, Washington,

DC). The “Adjust Threshold” function was used to obtain grain area. The threshold was adjusted until only grains were selected but any underlying cells were not, and a measurement of the selected area was taken. In order to obtain grain density, the threshold was adjusted again for the same image to select for the underlying cells and tissues as well (i.e. total area sampled), and a second measurement of the selected area was taken. The grain density was calculated as grain area/total area with cells sampled. It has been proposed that adjusting the threshold manually for each field of view in this manner, rather than using the same threshold measurement, gives more reliable results as it accounts for the variations in lighting and the staining density of the underlying tissue (Lewis et al., 1989, O'Shea).

2.5 LC-MS quantification of steroids

LC-MS was used for the quantification of steroids in Chapters 4, 5 and 6. The optimised LC-MS method is presented here, whilst the validation of the assay with quality control standards are presented in section 3.3.4, alongside the details of the development and optimisation process.

2.5.1 Steroid standards

Standards for corticosterone, 11-DHC, deoxycorticosterone (DOC), 5α-DHDOC, progesterone, 5α-DHP, allopregnanolone, pregnenolone, testosterone were purchased from Steraloids Inc (Newport, RI, USA) and 3α,5α-THDOC from Sigma (Leicester, UK), with catalogue numbers detailed in Table 3.1. All solvents/chemicals used were LC-MS grade. Individual steroid standards were dissolved in methanol (Honeywell Riedel-de Haën, Germany) into 1 mg/ml stock solutions, and were combined and diluted in methanol into a working stock solution containing 10 µg/ml of each steroid. Prior to each study, 10 µg/ml steroid standard mix was further diluted in 4% BSA into a stock solution containing 250 ng/ml of each steroid, and were aliquoted and frozen at -20^{o}C.

On the day of sample processing, one aliquot of the 250 ng/ml standard mix was further diluted 10-fold in 4% PBS, followed by a serial 2.5-fold dilution in 4% BSA to produce seven standard calibrants. The calibration standards used ranged from 102.4 - 25000 pg/ml for all steroid analytes (Table 3.5 and Figure 3.14). Samples from the same brain region were always run in the same batch, and each batch of samples were processed with seven standard calibrants and a zero sample containing only 4% BSA. The deuterated internal standards progesterone-D9 (Steraloids Inc), allopregnanolone-D5 (Tocris) and corticosterone-D5 (Sigma, Leicester, UK) were also diluted and combined from 1 mg/ml stock solution into 50% methanol/50% PBS.

2.5.2 Sample processing

Sample homogenisation: Frozen tissue samples (brain, placenta or liver) were weighed prior to sample processing, and transferred to Eppendorf tubes. For standard calibrants and plasma, 100 µL was aliquoted. Samples were homogenised in 500 µL of methanol/1% formic acid (FA) and sonicated on ice. For standard calibrants and plasma, 400 µL of methanol/1% FA was added. 20 µL of deuterated

internal standard mix (25 ng/mL of allopregnanoloned-d5, corticosterone-d4 and progesterone-d9; Table 3.1) were added and homogenates were briefly sonicated. After incubation on dry ice for 30 min to aid protein precipitation, homogenates were centrifuged for 10 min (13000 g at 4^{o}C). The supernatant was decanted into a borosilicate tube and the pellet was homogenised and sonicated again with another 500 µL of methanol/1% FA. After centrifugation, supernatants were combined, then diluted with LC-MS grade water to a final concentration of 30% methanol.

Solid phase extraction: Steroids in both plasma and brain samples were extracted by solid phase extraction using DSC-Discovery C18 100 mg columns (Supelco, UK). Columns were activated with 1 mL of methanol and equilibrated with another 1 mL of 30% methanol. Diluted supernatants from homogenates (ca. 3 mL) were then loaded, followed by two 1 mL washes of 40% or 50% methanol (40% methanol if 11-DHC was quantified). All steps were assisted by centrifugation at 50 g (average flow rate of 0.5 mL/min). Steroids were eluted with 1 mL of 85% methanol by gravity flow. The collected eluate was dried in a vacuum overnight. Dried samples were stored at -20^{o}C until the day of analysis.

Derivatisation: On the day of LC-MS analysis, 400 µL of freshly prepared derivatisation agent (1 mg/mL of Girard's T reagent, Sigma #89397; dissolved in methanol containing 0.2% formic acid) was added to the dried samples. After incubation at 37^{o}C for 30 min, the reaction was stopped by the addition of 50 µL of 5% ammonium hydroxide (ACROS Organics, New Jersey, USA) in methanol. Samples were dried in the SpeedVac then reconstituted in 50 µL of 50% methanol. The sample was transferred to Chromacol vials (ThermoScientific) for analysis.

2.5.3 LC-MS/MS analysis

Analysis of steroids was performed using an Ultimate 3000 Dionex HPLC system with a refrigerated autosampler, coupled to an AmaZon ETD ion trap mass spectrometer. Full details of the development, optimisation and validation of the method used is provided in Chapter 3. Briefly, separation of steroids on reverse phase HPLC was achieved on the ACE UltraCore 2.5 µM Super C18 column, maintained at 40^{o}C. Mobile phase A consisted of 50 mM ammonium formate pH 3 and while mobile phase B consisted of methanol with 0.1% formic acid, prepared freshly for each batch of analysis. Gradient separation was used, with gradient characteristics detailed in Figure 3.10. Steroids were analysed simultaneously using

multiple reaction monitoring, with positive electrospray ionisation and collision-induced fragmentation, and transitions monitored are detailed in Table 3.4. Injections for all samples, including calibrants, were carried out in duplicate.

Data was acquired using Hystar software and peak area under curve (AUC) was extracted and automatically integrated using QuantAnalysis v2.0 (both from Bruker Daltonics). The ratio of the peak AUC of the target analyte and the peak AUC of respective internal standards was used to construct the calibration curve. Concentrations of samples were extrapolated and converted to ng/mL (for plasma) or normalised to the wet weight of the tissues (ng/g; for brain tissues). All runs in this thesis are based on the assay performance characteristics documented section 3.3.4. A representative chromatogram is shown in Figure 3.9.

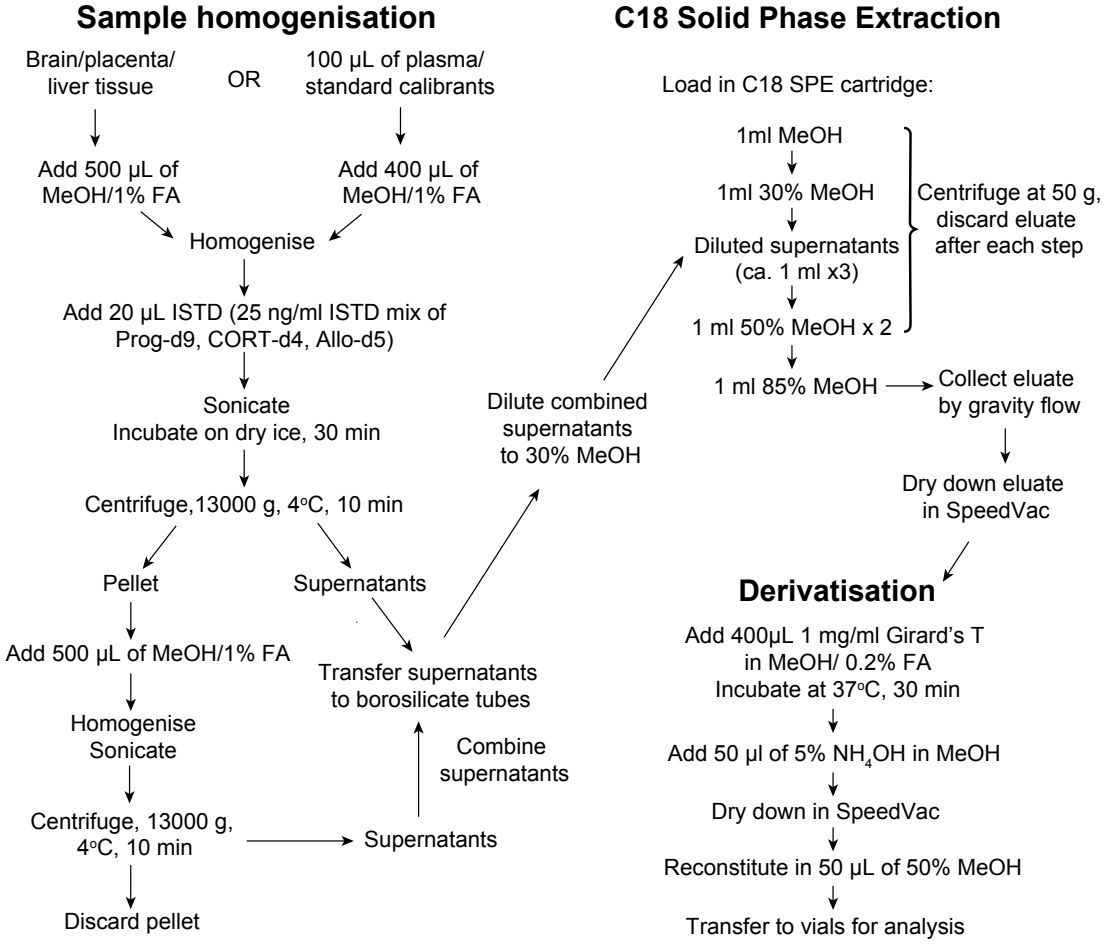


Figure 2.3: Schematic of LC-MS sample preparation procedure. Adapted from supplementary Fig S1 of Sze et al., 2018 (Appendix B).

2.6 Data analysis and statistics

All statistical analysis was performed using GraphPad Prism 6.0 or on R-studio (R scripts are attached in Appendix A). Graphical data are presented as group means with error bars representing the standard error of the mean (s.e.m.). Individual data points have also been overlaid on the bar graphs to show the spread of data and variation within a sample group.

Comparing between two groups

For the comparison of means between two groups, an unpaired Student's t-test was performed using GraphPad Prism 6.0. F-test for unequal variances was carried out and if variances were significantly different, a Welch's correction was performed. Significant differences ($p<0.05$) are annotated on the graphs, where * $p<0.05$, ** $p<0.01$, *** $p<0.001$, whilst t-values and degrees of freedom are reported in the text.

Comparing between more than two groups: ANOVA

For comparison of means between more than two groups, where there were two independent variables (e.g. prenatal stress status and acute stress in Chapter 4, sex and prenatal stress status in Chapter 5, and drug treatment and stress status in Chapter 6), two-way ANOVA was carried out using R-Studio (Appendix A). Normality and variance were first visually examined by the plotting of graphs of residuals, before performing ANOVA. Following which, post-hoc multiple pairwise comparisons was performed using the Student Newman Keuls post-hoc testing, also using R-studio. F-values and degrees of freedom of two-way ANOVA are reported in the text or under the graphs, whilst significant results from the post-hoc multiple pairwise comparisons were annotated on the graphs itself, using asterisks for effect of the first factor, and hashes for effect of the second factor. Significance level was set at 0.05 in all cases. There were instances in this thesis where there were three factors are involved in this study (e.g. acute stress, prenatal stress and sex in Chapter 4). These were analysed by three-way ANOVA and were also carried out using R-studio (Chapter 4, Appendix A and C), in consultation with Dr Crispin Jordan of the Centre for Discovery Brain Sciences, University of Edinburgh.

Chapter 3: Development and optimisation of a LC-MS method to measure steroids in rat brain tissues

3.1 INTRODUCTION

Given that this thesis is focussed on investigating the role of steroids in prenatal programming and stress-related disorders, a fundamental component of such studies is the ability to reliably measure steroid concentrations, especially in the tissues where they are expected to exert their effects (i.e. the brain). However, the physical and chemical nature of steroids and the complexity of biological tissues/fluids mean that this process is often fraught with difficulties (Taves et al., 2011, Wudy et al., 2018, Handelsman, 2017).

The following chapter first elaborates on the physical and chemical properties of steroids and the implications for their reliable quantification. A few common techniques in the quantification of steroids are then introduced, and for each case, their limitations are also considered. As this thesis relies on LC-MS as the primary method for steroid quantification, the basic principles of LC-MS will be explained. The chapter then details the process of developing and validating a reliable method for the analysis of biological compounds such as steroids using LC-MS. This process will then be applied the development of a LC-MS method to quantify a list of steroids, namely corticosterone, 11-DHC, DOC, DHDOC, THDOC, progesterone, DHP, allopregnanolone, testosterone, DHT, 3α-diol, 3β-diol and oestradiol (briefly introduced in Section 1, Aim 1). The chapter then ends with a study involving the administration of an acute stressor (i.e. swim stress) to male and female adult rats, which is known to increase neurosteroid levels in the brain and plasma, to biologically validate the LC-MS method.

3.1.1 General structure and properties of steroids

All steroids, natural or synthetic, consist of a four-carbon ring core (three six-carbon rings A, B and C; and 1 five-carbon ring, D). All steroids have 21 or fewer carbons, and are all derivatives of cholesterol (C27)(Fig 3.1). The first rate limiting step in steroid synthesis involves the cleavage of the bond between C20 and C22 catalysed by the enzyme p450scc, leading to the removal of 6 carbon atoms from cholesterol into pregnenolone (C21) (Fig 3.1) (Berg et al., 2002). Steroid hormones can be classified according to the number of carbon atoms they possess and their biological function into four major groups, the progestogens (C21), adrenocorticoids (C21), androgens (C19) and oestrogens (C18) (Figure 3.2). Within each class, individual steroids differ by the location, type and configurations of their functional groups,

which determine their overall 3D conformation and their physical and chemical properties. These in turn determine their interactions with receptors, co-chaperones and enzymes, which ultimately contribute to their biological functions. In the context of this chapter, these physical and chemical properties also contribute to the complexities in the method used to extract, identify and measure them.

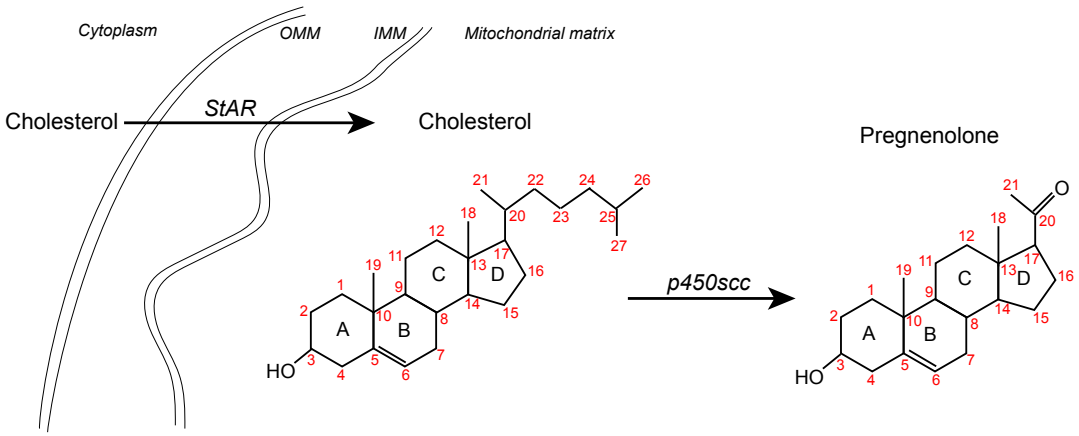


Figure 3.1: Side chain cleavage of cholesterol. The first and rate-limiting step of neurosteroidogenesis consists of the cleavage of cholesterol (C27) into pregnenolone (C21), the precursor of all neuroactive steroids, catalysed by the enzyme cytochrome p450 side chain cleavage enzyme (p450scc). Steroidogenic acute regulatory protein (StAR) facilitates the transport of cholesterol from outer mitochondrial membrane (OMM) into the mitochondrial matrix, where p450scc acts (Miller, 2007). IMM: Inner mitochondrial membrane.

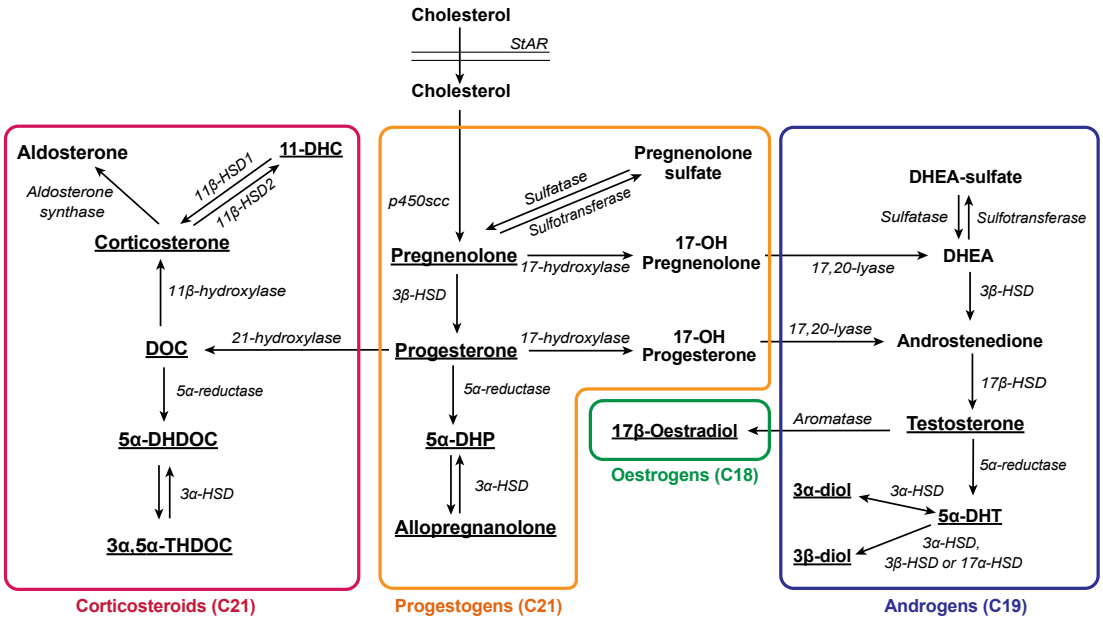


Figure 3.2 The different major classes of steroids. Several of the key steroids which are involved in modulating stress responses are selected for quantification in this chapter are underlined. They include corticosterone, 11-dehydrocorticosterone (11-DHC), deoxycorticosterone (DOC), dihydrocorticosterone (DHDOC), tetrahydrocorticosterone (THDOC), progesterone, testosterone, dihydrotestosterone (DHT), 3α-androstanediol (3α-diol), 3β-androstanediol (3β-diol), allopregnanolone, pregnenolone, and 17β-Oestradiol. Other abbreviations: DHEA, Dehydroepiandrosterone; HSD: Hydroxysteroid dehydrogenase, StAR: Steroidogenic acute regulatory protein.

All natural steroids have an oxygen function at C3 (Fig 3.3), which could exist as a hydroxyl group (C-OH; in the α or β configuration), as a phenolic hydroxyl group attached to an aromatic A ring (e.g. in the oestrogens) or as a carbonyl group (C=O) where oxygen is double bonded to the C3 carbon (Kasal, 2010). This functional group at C3 is of particular interest, due to the metabolic reactions that rely on it, catalysed by the enzymes 5α- and 5β-reductase, and 3α- and 3β-hydroxysteroid dehydrogenase (HSD). 5α and 5β-reductase targets steroids with a C3 ketone group (C=O) and a C4-C5 double bond (i.e. 3-keto-$\Delta^{4,5}$ steroids). The C4-C5 double bond is broken and the unsaturated carbonyl group is reduced, which can allows the C3 ketone group to be further reduced to a saturated alcohol by 3α- or 3β-HSD. These two reactions, illustrated in Fig 3.3, are important in the metabolism of many sex hormones and neuroactive steroids. Each of these reactions alter the structure of the steroid, and therefore their binding properties to receptors. For instance, the unsaturated pregnenolone sulfate is planar and potentiates NMDA receptors, while

the saturated, 3α,5β-reduced pregnanolone sulfate has a *cis* junction resulting in a "bent" structure, and instead acts as an inhibitor of NMDA receptors (Weaver et al., 2000).

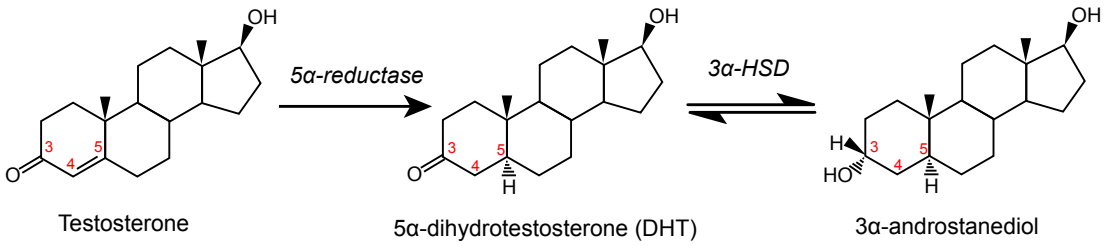


Figure 3.3: 5α and 3α reduction of steroids. 5α-reductase targets 3-keto- $\Delta^{4,5}$ steroids (such as testosterone in this example) with a C3 ketone group (C=O) and a C4-C5 double bond, and irreversibly breaks the $\Delta^{4,5}$ bond in a stereospecific manner, inserting a hydride ion (H-) at the alpha face of C5 (Jin and Penning, 2001). This reduction rearranges the structure of the rings of the steroid, allowing it to be further reduced by 3α-HSD into 3α-diol in this case.

Additionally, the presence of α- and β-configuration, which refer to the functional group being below and above the plane of the steroid backbone, means that there are four possible stereochemical configurations that can exist for saturated steroids. Stereoisomers have the same molecular weight, but differ in their physical and chemical properties, which have implications in their biological activity. For instance, the spatial configuration of the C5 functional group is especially important for the potency of steroids on $GABA_A$ receptors and their function as anaesthetics as they determine cis-trans isomerism at the A:B steroid rings (Covey et al., 2000). The α orientation at C5 yields a A:B *trans* isomer with a nearly planar structure, whereas β orientation gives a A:B *cis*-isomer with a bent structure (Berg et al., 2002). Metabolites with 5α-reduction (e.g. allopregnanolone and 3α,5α-THDOC) generally have more potent agonist actions than its 5β-isomer (e.g. pregnanolone and 3α,5β-THDOC respectively) (Mennerick et al., 2004, Gee and Lan, 1991). Methods used to quantify steroids would therefore need to have the selectivity to differentiate these stereoisomers.

Given their hydrocarbon skeleton, another physical property of steroids is that they are largely hydrophobic in nature and are therefore found in biological fluids either bound to proteins (i.e. binding globulins) or exist in conjugated forms (Makin, 2010).

As mentioned in section 1.3, steroids can bind to corticosterone corticosteroid-binding globulin (CBG) and sex hormone-binding globulin (SHBG) with high affinity, and can also bind non-specifically to albumin, which is present in the plasma in large amounts (Makin, 2010). Steroids can also form conjugates by the modification of functional groups, such as through the addition of a glucose or sulfate group, which renders them more hydrophilic (Makin, 2010).

These physical and chemical properties have certain implications for the extraction and quantification of steroids. Firstly, given their hydrophobicity, steroids are more soluble in organic solvents rather than in aqueous solvents, thus their extraction solvent needs to be carefully considered. However, their varying degrees of hydrophobicity (and therefore solubility in different solutions) may also serve as an advantage which may aid their separation and identification, especially in the case of LC-MS. Secondly, the existence of binding globulins calls for the need to differentiate between “free” and “total” amounts of steroids quantified, which has implications for the method of sample processing chosen.

3.1.2 Immunoassay methods for quantifying steroids

The quantification of steroids has traditionally been carried out using immunoassays, which includes both radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA). Immunoassays rely on the specificity of antibody binding to quantify steroids of interest and can often be conducted without the need for extraction or purification of biological fluids. In recent decades, kits that contain all the necessary reagents to perform these immunoassays have been developed commercially, making them convenient and accessible to researchers (Stanczyk et al., 2007).

However, there are some disadvantages associated with using immunoassays to quantify steroids, with the most familiar criticism being the assay's proneness to cross-reactivity (Selby, 1999, Klee, 2004). Antibodies, especially polyclonal antibodies, may have varying degrees of specificity for their antigen, and some may also bind to molecules other than the target molecule. This can be problematic for steroids, due to their close structural similarities. For instance, an antisera against progesterone that was generated in rabbits was found to cross-react with its metabolites 5α- and 5β-DHP, allopregnanolone, epi-allopregnanolone and epi-pregnanolone (Murphy and Allison, 2000). Secondly, although multiplexed immunoassays have been developed in recent years for other analytes such as cytokines, this is not the case for steroids, due to their propensity for cross-reactivity. Steroid immunoassays therefore generally do not allow for the quantification of more than one type of steroid hormone in a single analysis, which limits the utility of the method, especially if a more complete picture of the steroidal milieu is required (Taylor et al., 2015). For such instances, detection of steroids using mass spectrometry (next section) offers much more potential and is the method of choice.

Nonetheless, for laboratories with no significant capabilities to own a mass spectrometer, immunoassays are accessible and thus still have value in many studies today, especially for the routine quantification of single steroids that are present in high concentrations in the plasma (e.g. cortisol or corticosterone) (Wudy et al., 2018). In this thesis, both immunoassays and LC-MS methods were used, as some studies were carried out before the LC-MS method in this chapter had been fully developed.

3.1.3 Mass spectrometric quantification of steroids

3.1.3.1 Basic principles of mass spectrometry and chromatography

Mass spectrometry (MS) separates compounds based on their mass-to-charge ratio (m/z). It functions on the basic principle that when a charged particle moves through either a magnetic or electric field, the deflection of its path is dependent upon both the mass and charge of the particle (i.e. heavier and less charged molecules deflect less) (McDonald et al., 2011).

There are three basic components in a mass spectrometer, (i) the ion source where analytes are ionised, (ii) the mass analyser where ions are separated according to their m/z ratio, and (iii) the detector and data system where an output is given as a graphical form, with ion intensity plotted against m/z ratio. In biological applications, tandem mass spectrometry (MS/MS) is often used, where more than one stage of mass analysis occurs, adding another level of selectivity in the method (Soldin and Soldin, 2009, Kushnir et al., 2005). MS/MS involves the first step of detecting and isolating the precursor ion (MS1), then fragmenting it into product ions (MS2) (Fig 3.4). A common method of fragmentation, especially in small molecule analysis, is collision-induced fragmentation, where inert gases such as argon and nitrogen are used to break down molecules at weak points in their chemical structure. Different molecules of the same molecular mass can fragment differently, and the fragmentation patterns can aid the identification of the molecule.

However, differentiating compounds simply by their m/z, even with fragmentation, is insufficient, especially in the case of steroid stereoisomers, as they are likely to have the same fragmentation pattern given their similarity in structure (Kushnir et al., 2005). As such, an additional separation step is often required before introducing the sample into the mass spectrometer, e.g. the use of chromatographic applications such as gas chromatography (GC) or liquid chromatography (LC). Chromatography is the technique of separating components in a mixture, and relies on two phases, the “mobile phase” and the “stationary phase” (Hage, 2018). The components of the mixture are carried in the mobile phase and interacts with the stationary phase, which usually exists in the form of a solid support such as a column. Separation is based on the affinity the components have for the mobile or stationary phase, and components that interact strongly with the stationary phase will move through the column more slowly (Fig 3.5). On the other hand, components

which have weaker interactions with the stationary phase will move through the system quickly due to lower retention. The resulting outcome is that different components of a biological mixture will have different rates of travel through the chromatographic system (Hage, 2018). Combinatorial techniques, like GC-MS or LC-MS, thus allows for different analytes to enter the mass spectrometer in a time-staggered manner, before undergoing ionisation and fragmentation as described in Fig 3.4. Thus, even if more than one component in the mixture has the same mass and fragmentation patterns, they can be differentiated by their distinct retention times. By combining the separation power of chromatography, and the selectivity of MS, a potentially powerful method can be generated to identify and quantify hundreds of small, structurally-related compounds, including steroids.

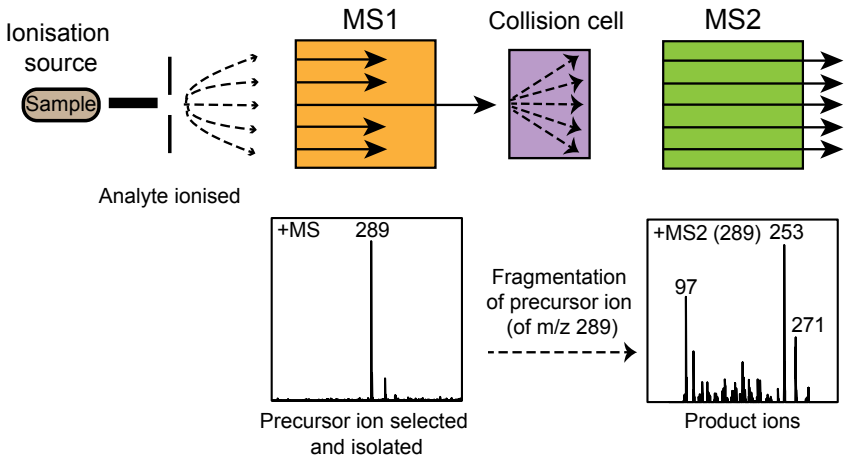


Figure 3.4: Basic principles of tandem MS/MS. Using the example of testosterone, which is the precursor ion and has a m/z of 289 and is isolated at MS1. Following fragmentation, product ions with m/z 97, 253, and 271 are being produced, and the intensity of these product ions are being quantified. These product ions are the signature fragmentation pattern of testosterone, and molecules that are of m/z 289 but do not fragment in this manner (i.e. contaminants) are excluded from quantification, adding another layer of selectivity to the method.

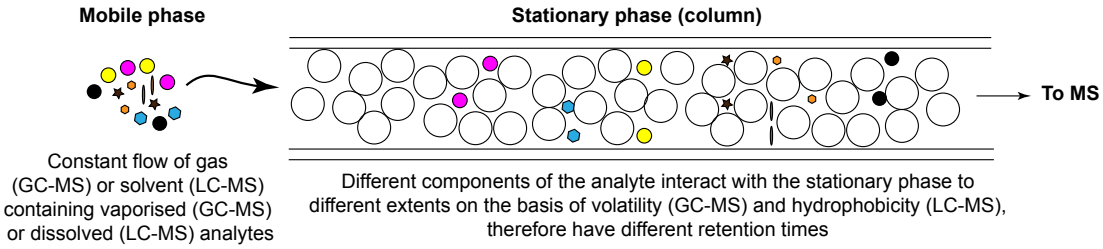


Figure 3.5: Basic principles of chromatography. Both GC-MS and LC-MS consists of a mobile phase and a stationary phase, and is based on the basic principle of differential interactions of different compounds of the analyte with the stationary phase.

3.1.3.2 GC-MS quantification of steroids

In gas chromatography, components of the analyte mixture are vaporised into an inert carrier gas which acts as the mobile phase. Separation of components of an analyte are based on their volatility and their interactions with the stationary phase, usually a solid column or one that is coated with a liquid. More volatile components spend more time in the mobile phase, therefore elute more quickly.

The advent of GC-MS was a major breakthrough in the study and quantification of steroids in the 1960s. Although often regarded as the older technique, GC-MS technologies are constantly being improved, and optimised GC-MS methods offer very good resolution and sensitivity and are widely used in the field of endocrinology (Shackleton et al., 2018, Krone et al., 2010). Several published GC-MS methods have been used to reliably quantify neuroactive steroids in the brain, where they were able to successfully distinguish between structural and stereoisomers with good detection limits. For example, using GC-MS, Vallée and colleagues have shown that testosterone, DHEA, pregnenolone, allopregnanolone and its isomer isoallopregnanolone could be quantified to a level of < 0.5 ng/g in male rat frontal cortex (Vallee et al., 2000). Another group has shown that in the rat prefrontal cortex and hippocampus, GC-MS methods could similarly detect pregnenolone and allopregnanolone at the lowest limit of quantification (LOQ) of 0.5 ng/g in the male rat brain (Servatius et al., 2016).

However, the drawback of GC-MS is that it is labour-intensive, requiring extensive sample work-up in order for steroids to become volatile (McDonald et al., 2011,

Shackleton et al., 2018). Sample derivatisation, which involves chemically modifying the analyte to allow for analytes to be vaporised, is an absolute necessity in GC-MS, and is often seen as a disadvantage as it increases sample processing time and adds considerable variability to the method. The nature of sample processing in GC-MS also necessitates the hydrolysis of conjugated groups (such as glucoronides and sulfates), limiting the use of GC-MS in the study of conjugated steroid metabolites (McDonald et al., 2011). As such, this led to the next era in steroid quantification, where LC-MS was gradually introduced.

3.1.3.3 LC-MS quantification of steroids

LC-MS differs from GC-MS by the use of a liquid solvent as the "mobile phase", and molecules are separated based on polarity differences and its solubility in the carrier solvent. In steroid analysis, "reverse phase" LC is the preferred method, where the "stationary phase" consists of a LC column containing silica linked to hydrocarbon tails (C18), rendering them non-polar/hydrophobic. The speed at which components of the mixture travel through the column are based on their hydrophobicity, where more hydrophobic components interact more strongly with the material in the column, and therefore have longer retention times.

The most direct advantage of LC-MS is that it allows for the analysis of non-volatile compounds, and does not always require sample derivatisation, leading to the relative ease of sample preparation. Another advantage of LC is that it consists of a high-pressure pump system that is capable of mixing two solvents, therefore has the ability to change the mobile phase polarity over the course of the analysis. This allows for shorter run times as compared to GC, and also more flexibility in determining how analytes of interest are eluted, therefore can be considered to have higher throughput (Taylor et al., 2015). LC-MS has been successfully applied to the quantification of many steroids in a variety of different tissues with high sensitivity, even without sample derivatisation. For instance, Caruso and colleagues managed to detect 9 steroids with a LOQ ranging from 0.02 – 0.10 pg per sample in the rat brain without derivatisation (Caruso et al., 2010). A LC-MS method quantifying a panel of 12 steroids was also applied to human serum samples, with limits of detection (LOD) that range from 1.5 to 10 pg/mL (Guo et al., 2006).

However, to date, due to the complexity of the composition of brain tissue and the differing capabilities of MS instrumentation in different facilities (for example,

different modes of ionisation), a substantial number of analyses involving neuroactive steroids require derivatisation (Higashi and Shimada, 2004, Higashi and Ogawa, 2016). For electrospray ionisation (ESI) especially where analytes are ionised by applying a strong electrical current, derivatisation aids the generation of charged ion due to the permanent charge present in derivatisation agents (Higashi and Ogawa, 2016). Derivatisation with 2-hydrazino-1-methylpyridine (HMP) for example improved LOQ of allopregnanolone, epi-allopregnanolone and 5α-DHP 60- to 150-fold lower as compared to a method without derivatisation (Higashi et al., 2007). More recently, Dury et al. also managed to quantify several key neurosteroids in the monkey brain, with Girard's T derivatisation (Dury et al., 2016).

Despite being a powerful tool, LC-MS is subject to pitfalls and may generate erroneous data if there is inadequate sample workup or inappropriate choice of procedures (Liere and Schumacher, 2015, Vogeser and Seger, 2010). Furthermore, even if similar methods are developed elsewhere, specific differences or limitations in instrumentation between laboratories mean that one assay might not be readily applied in another laboratory without significant method development and optimisation (Liere and Schumacher, 2015). For every new in-house LC-MS assay, an analytical workflow must thus be properly delineated and followed, which is covered in the next section.

3.1.4 Developing and optimising a LC-MS method for the quantification of steroids

The LC-MS method development process is a reiterative one, and a summary is presented in Fig 3.6 based on published guidelines (Sargent, 2013) and other articles (Honour, 2011, Makin, 2010, Nair, 2017). Decision points exist at several stages of the workflow and often, compromises and trade-offs will have to be made depending on logistical, technical or time constraints. Eventually, the finalised method will have to balance efficacy, speed and cost (McDonald et al., 2011).

The process begins with the first step of MS optimisation, where precursor and product ions are identified, and fragmentation parameters are determined. Analytes that are able to ionise and fragment adequately are then brought forward to the next step for LC optimisation. The main aim of LC optimisation is to ensure that there is adequate separation of chromatographic peaks, so that different analytes enter the MS in a staggered manner. Along with the choice of column, the composition of the mobile phase and the gradient profile during the run will also have significant impacts on resolution and peak shapes, and need to be carefully considered. Upon satisfactory results at the end of this stage, the LC and MS aspects are combined and a preliminary LC-MS method with multiple reaction monitoring (MRM) is developed, where an entire panel of steroids can be detected and quantified with one single sample injection.

Using this preliminary LC-MS method, the sensitivity of the assay is then investigated. If the sensitivity of the assay is not satisfactory at this point (i.e. not close to physiological concentrations), derivatisation will need to be carried out to improve signals. Derivatisation, as mentioned earlier, chemically changes the functional groups of analytes. This effectively creates a new compound for analysis, and the process of MS tuning and LC optimisation needs to be repeated from the first step again. Several derivatisation agents may need to be screened until an optimal one is found, whilst derivatisation conditions will also need to be optimised to ensure maximal yield and stability of the derivative.

Once the sensitivity of the chosen method is established, it can subsequently be tested on biological tissues, where matrix effects may come into play. The term “matrix effect” refers to the alteration of the intensity of the MS response of a target analyte in a biological matrix (such as plasma or brain tissue) as compared to its

response in a calibrant solution (Panuwet et al., 2016, Taylor, 2005). This is due to the presence of co-eluting matrix components (e.g. lipids) which can affect the behaviour of target analytes of interest. The presence of interfering compounds at high concentrations could mask the detection of target analytes which tend to be of low abundance (Keshishian et al., 2007), compete with target analytes for charges during ionisation (Panuwet et al., 2016), or even react with target analytes to change their physical or chemical properties (Gosetti et al., 2010). These alterations may either decrease (i.e. ion suppression) or increase (i.e. ion enhancement) the signal of the target analyte, leading to inaccuracies in quantification. This is an especially pertinent issue for brain tissues, which is a complicated matrix given its high lipid and protein content in comparison to that of steroids (Taves et al., 2011).

One of the strategies to mitigate matrix effects is to include appropriate sample clean-up procedures in the quantification method. The aim of sample clean-up is to ensure that interfering substances are removed, whilst analytes of interest are retained. Sample clean-up also ensures that a relatively “clean” sample is injected into the LC column and mass spectrometer, protecting the instruments in the long run. Solid-phase extraction (SPE) is by far the most common method of sample clean-up, where it exploits the same principles of analyte and solid phase interactions as in LC (Campíns-Falcó et al., 2012). The solid phase is however, packed in smaller one-use cartridges with greater particle sizes, and is a far more rudimentary form of separation as compared to the LC column. Various types of SPE cartridges are available commercially, the choice of which is dependent on the characteristics of the analyte of interest and also the biological matrix.

The addition of internal standards can also help to decrease the impact of matrix effects, as they can compensate for variations in MS and LC conditions, and also errors in sample preparation. An internal standard is a compound that is added in a known amount to every sample, and should be similar but not identical to the analyte of interest. Its chemical and physical properties should resemble the analyte, such that it behaves similarly to the analyte of interest in all aspects of the LC-MS method (Makin, 2010). In the LC, it should be well-resolved from other peaks, but have similar retention to the column as the analyte of interest, whilst in the MS, it should have similar ionisation and fragmentation patterns (Sargent, 2013). The ratio of the signal response between the internal standard and that of the analyte of interest is used for plotting the calibration curve. Therefore, the idea is that in cases

where there is interference from the matrix or if random experimental errors arise, both the internal standard and analytes will be affected in the same manner and to the same extent, and the ratio remains unaffected. The selection of an appropriate internal standard is therefore a major part of the method development process. After the selection of appropriate internal standards, known concentrations of internal standards should then be incorporated to all standard calibrants and tissue samples prior to sample processing.

Once a reasonable method which takes into account aforementioned two points is developed, it has to undergo a full validation procedure before it can be implemented in biological studies (Sargent, 2013). If the accuracy and precision of the method are within acceptable limits, bioanalytical results can then be considered reliable. Conversely, poor validation results can reveal problems in the method and/or instrumentation that need to be addressed. Ultimately, if the method is to be established as a routine assay, the validation should also be carried out in a routine manner. As such, the method development and optimisation process can be considered a continuous one, involving troubleshooting and also modifications which can lead to constant improvements in the assay.

Lastly, as an extension of the validation process, the biological verification of the assay can additionally be carried out. Biological verification demonstrates the assay's ability to detect biologically relevant differences between treatment groups. In the context of this study, the biological verification of the assay would be the ability to detect differences in steroid concentrations between samples that are expected to show significant differences in steroid concentatrations (for instance, greater testosterone concentrations in male plasma as compared to female plasma, or greater corticosterone concentrations following a stressor).

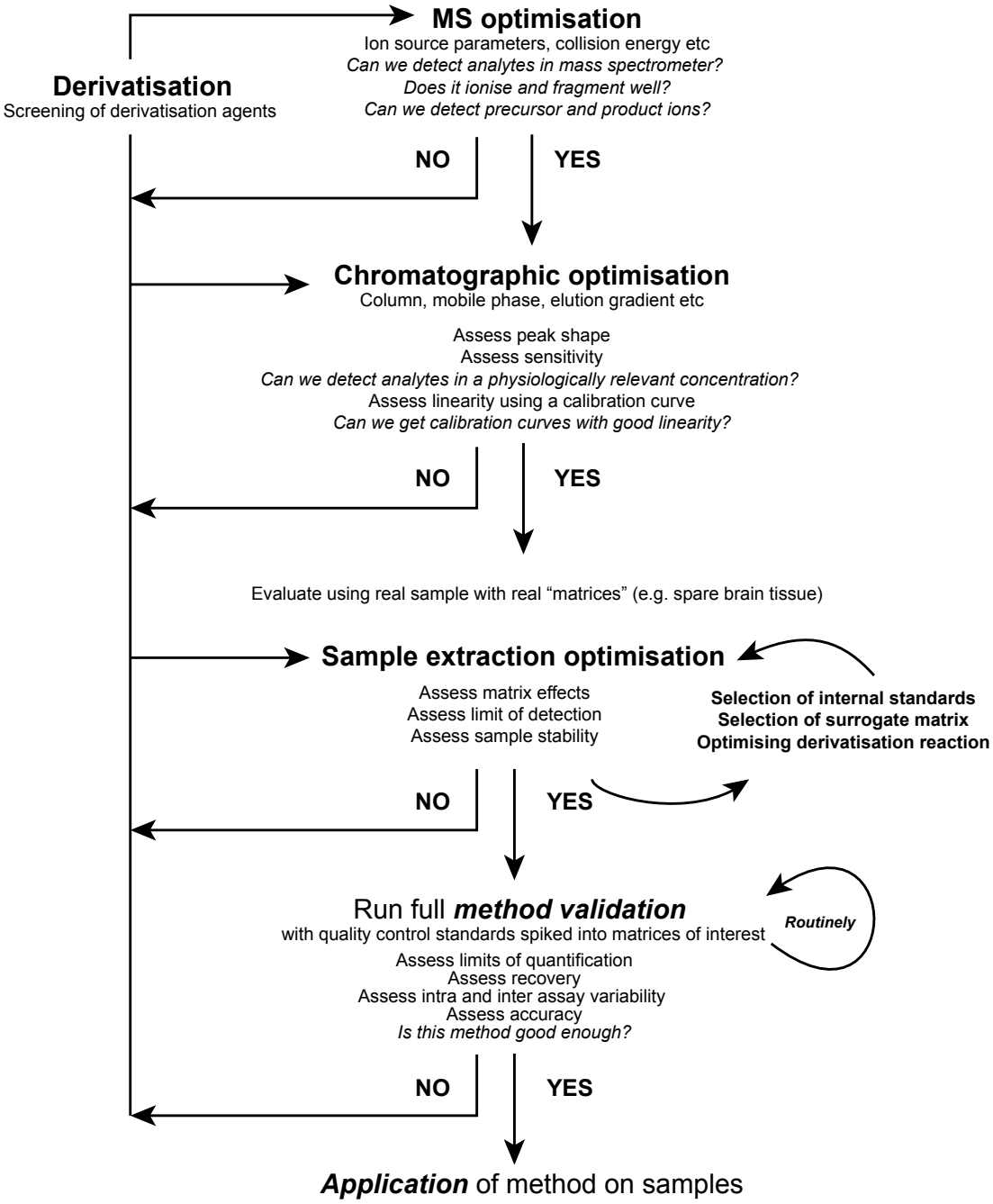


Figure 3.6: Workflow for the development and validation of a LC-MS method

3.1.5 Aims

The work in this chapter aims to:

1. Develop a method to quantify as many as possible of these 14 steroids of interest in a single analysis: Corticosterone, 11-dehydrocorticosterone (11-DHC), DOC, DHDOC, THDOC, Progesterone, 5α-Dihydroprogesterone (DHP), Allopregnanolone, Pregnenolone, Testosterone, 5α-Dihydrotestosterone (DHT), 3α-androstanediol (3α-diol), 3β-androstanediol (3β-diol) and 17β-Oestradiol using the workflow in section 3.1.4. As it is expected that not all of these steroids may be successfully quantified in a single method, several steroids that are deemed to be more heavily involved in the modulation of the HPA axis (e.g. corticosterone, and the positive $GABA_A$ modulatory steroids allopregnanolone and THDOC) are prioritised.

2. Validate the method as closely as possible according the guidelines for bioanalytical methods issued by the Royal Society of Chemistry (Sargent, 2013). This includes assessment of the linearity of standard curves, limit of quantification (LOQ), limit of detection (LOD), recovery percentage, measurement accuracy, intra- and inter-assay variability for all steroids.

3. Biologically validate the developed method and demonstrate that the LC-MS method is sensitive enough to detect significant differences in steroid concentrations between different treatment groups. In order to achieve this aim, steroid concentrations were measured in the plasma and brains of male and female rats, with and without acute stress (swimming stress). The study aims to assess the ability of the LC-MS method to (i) replicate expected patterns of increase in neuroactive steroids following stress (for instance, an increase in neuroactive steroids such as corticosterone and allopregnanolone in the plasma and the brain following acute stress), (ii) show expected differences in sex hormone concentrations between males and females (for instance, testosterone is greater in males as compared to females). Additionally, this study also aims to assess the feasibility of using swimming stress as a form of acute stress to elicit a response involving several neuroactive steroids not previously investigated in other studies, thereby laying the foundation for the designing of studies incorporating acute and prenatal stress in the subsequent chapter.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

All steroid standards and internal standards were purchased in solid form from either Steraloids Inc. (Newport, RI, USA), Sigma (Leicester, UK) or Tocris Bioscience (Bristol, UK) (Table 3.1). The structure of the steroids as well as the deuterated internal standards are presented in Figure 3.7. All solvents/chemicals used were LC-MS grade. Methanol was purchased from Honeywell Riedel-de Haën (Seelze, Germany), whilst LC-MS grade water, formic acid (FA; LC-MS Optima) and ammonium formate powder were procured from Fisher Scientific (Leicester, UK).

Steroid standards were carefully weighed, dissolved in methanol and stored in -20^{o}C as 1 mg/mL stock solutions.

Steroid	**CAS no.**	**Catalogue no.**
Corticosterone	50-22-6	Steraloids; Q1550-000
11-dehydrocorticosterone	72-23-1	Steraloids; Q3690-000
DOC	64-85-7	Steraloids; Q3460-000
5α-DHDOC	298-36-2	Steraloids; P3750-000
3α,5α-THDOC	567-02-2	Sigma; #P2016
Progesterone	57-83-0	Steraloids; Q2600-000
5α-Dihydroprogesterone (DHP)	566-65-4	Steraloids; P2750-000
Allopregnanolone	516-54-1	Steraloids; P3800-000
Pregnenolone	145-13-1	Steraloids; Q5500-000
Testosterone	58-22-0	Steraloids; A6950-000
5α-Dihydrotestosterone (DHT)	521-18-6	Steraloids; A2570-000
3α-androstanediol (3α-diol)	1852-53-5	Steraloids; A1170-000
3β-androstanediol (3β-diol)	571-20-0	Steraloids; A1220-000
17β-oestradiol	50-28-2	Steraloids; E0950-000
Progesterone-D9		Steraloids; Q2600-014
5α-dihydroprogesterone-D8		Steraloids; P2750-030
Allopregnanolone-D5		Tocris Bioscience; #5532
Corticosterone-D4		Sigma; #802905

Table 3.1: Steroid analytes used and tested in this thesis and associated CAS numbers and catalogue numbers. Deuterated internal standards are in grey.

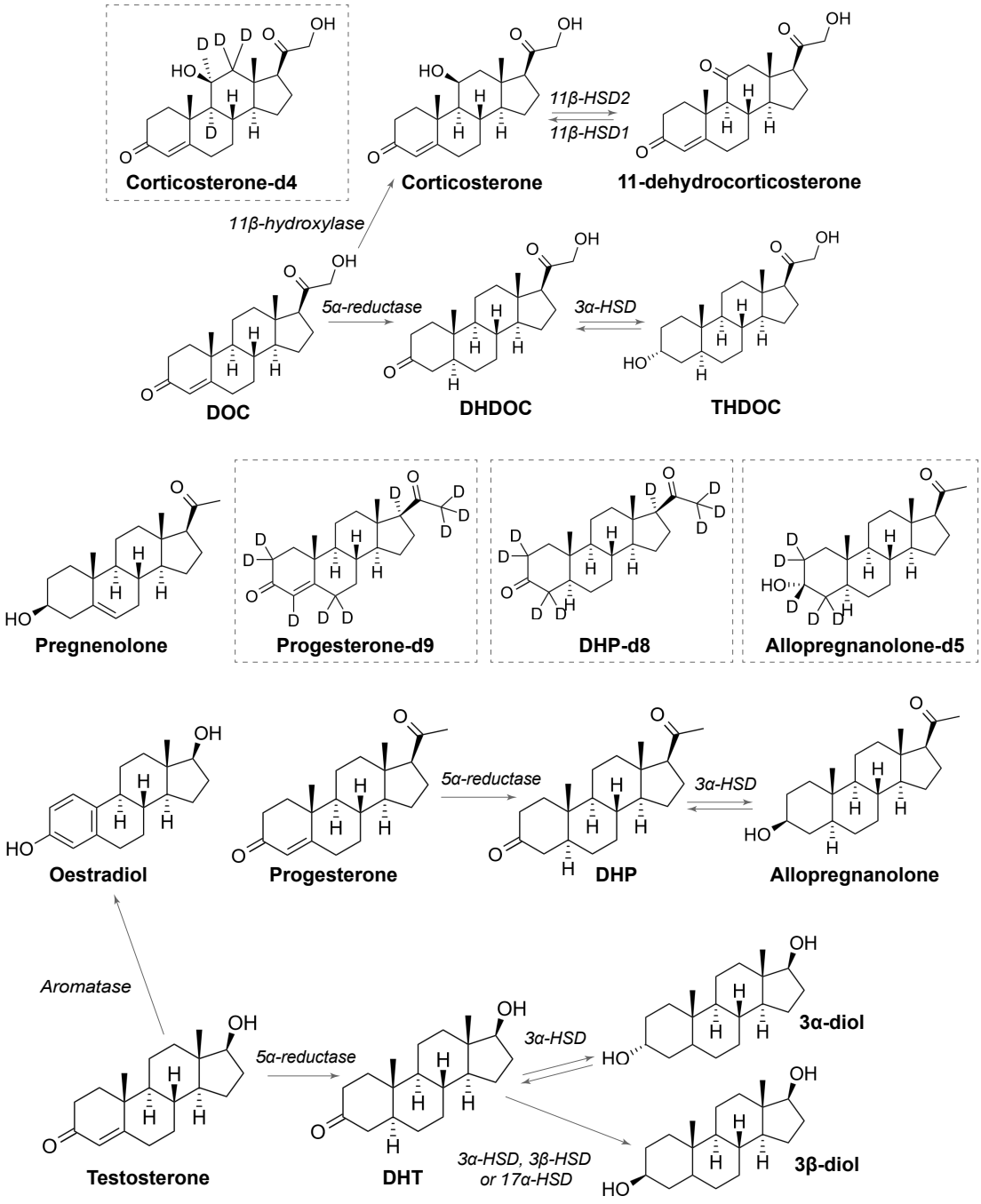


Figure 3.7: Structure of the steroid analytes investigated in this study. The steroids differed from each other by functional groups, and are interconverted by steroidogenic enzymes. Internal standards are also shown (in the dotted boxes) to illustrate the position of their deuterium substitutes.

3.2.2 Instrumentation and software

All LC-MS experiments were carried out in the Roslin Institute Proteomics and Metabolomics Facility, in collaboration with Dr Andrew Gill.

MS was carried out on the amaZon ETD ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) and the mode of ionisation was ESI. Operating conditions for MS was capillary temperature of 200°C, capillary voltage of -4500V, nitrogen nebuliser gas at 16 psi, dry gas at 8L/min and dry temperature of 150^{o}C. Argon was used as the collision gas.

LC was carried out on the Ultimate 3000 Dionex HPLC system, with a refrigerated autosampler maintained at 8^{o}C. The injection volume was 5µL, with a partial loop injection. The syringe was rinsed with 20% methanol after each sample injection.

The TrapControl software was used for MS tuning while Hystar software was used for LC-MS. Spectral data was viewed and visually compared on DataAnalysis software. Following LC-MS, the peak area under the curve was extracted and automatically integrated using QuantAnalysis v2.0 (all software from Bruker Daltonics). Each chromatogram was visually inspected to ensure that AUC was correctly integrated and that there were no interfering peaks.

3.2.3 MS optimisation methodology

Mass spectrometry detection was the first procedure to be optimised. Solutions consisting of individual steroids were prepared separately in methanol (5 µg/mL) and infused directly into the source via a Hamilton syringe pump at a rate of 5 µL/min. Scanning for the precursor ion (MS1) was first carried out using the UltraScan resolution as scan mode (32500 m/z per second). Fragmentation mode (MS2) was then enabled and fragmentation conditions (amplitude and cut-off) were optimised using the SmartFrag module in TrapControl. This was carried out for both underivatised steroids at the early stages of the optimisation process, as well as the various derivatised steroids at the later stages.

3.2.4 LC optimisation methodology

Column: ACE UltraCore 2.5 µM Super C18 column (75 mm by 2.1 mm i.d.; #CORE-25A-7502U, Advance Chromatography Technologies, Aberdeen, UK) was selected as the column of choice based on a literature review where C18 columns were used

for various studies (Taylor et al., 2017, Ma and Kim, 1997, Jin et al., 2013, Rustichelli et al., 2013) as well as its solid core technology which allows for better separation without increasing back pressure on the system (Ahmed et al., 2018). The flow rate of 0.2 ml/min was selected to match its internal diameter of 2.1 mm. The column was maintained at 40^{o}C. Columns were replaced every 5000 injections, and were regenerated via a reverse flow wash protocol comprising of methanol, acetonitrile, acetronitrile/isopropanol (3:1 v/v), isopropanol, dichloromethane, hexane in succession when the back pressure of the system became too high.

Mobile phase: For mobile phase A (the aqueous/ more polar component), (i) 50 mM ammonium formate (pH 3.0) and (ii) water (with 0.1% FA) were tested while for mobile phase B (the organic/ less polar component), (i) methanol and (ii) acetonitrile, both with 0.1% FA, were tested. Mobile phase optimisation was carried out in the initial stages using underivatised steroids in MS1 mode, using a gradient protocol where the % of B was increased gradually from 50-100%, with a total run time of 10 min. AUC of extracted ion chromatograms (EIC) at MS1 were compared for the different combinations of mobile phase A and B. Chromatograms were also compared for the best peak shapes, where parameter such as peak intensity and peak symmetry were compared visually (Sargent, 2013). A good peak should have reasonable peak intensity (a signal to noise ratio of at least 10^3 for a steroid with 5 µg/mL concentration), is symmetrical, with minimal peak broadening, or peak fronting and tailing at the base (Sargent, 2013). The combination of 50 mM ammonium formate (A) and methanol/0.1% FA (B) gave the highest peak intensity and reasonable peak shapes, and was thus selected as the mobile phase. This mobile phase combination was used for all subsequent optimisation protocols with derivatised reagents. All solvents were prepared fresh prior to a run and were degassed for 20 min in a water-bath sonicator, which aids the consistency of retention time.

Gradient optimisation: Steroids were first individually injected into the LC to determine the retention time. The relative composition (%) of solvents A and B during the course of the run was then altered to ensure that each specific steroid of interest eluted with a different retention time. This was first carried out using MS1 mode, with a high concentration of steroids (5 µg/mL, similar to direct infusion levels). Once the retention time was determined, MS2 mode was carried out, where the efficacy of fragmentation was re-assessed with the mobile phase composition

taken into consideration. Chromatograms were again visually inspected here for reasonable peak shapes. Additionally, an equilibration step prior to sample injection (maintained at 5% B), and a washing step after the end of the run (maintained at 100% B) was also added to aid the consistency of the elution time and to prevent carry-over effects. A divert valve was also used in which contaminants eluting before the gradient reached 40% B, and after 100% B were being diverted to waste so as to not overload the electrospray nozzle.

Preliminary LC-MS method with multiple reaction monitoring (MRM): Once a satisfactory gradient profile was constructed, the LC method and the MS method were then combined into a single preliminary LC-MS method, with multiple reaction monitoring. The method was split into several time segments, with each segment detecting specific MRM transitions (not more than 5 per time segment), allowing the detection of more than one analyte in a single injection. Steroid standards were then combined into a single sample, serially diluted, and analysed using this preliminary LC-MS method to establish the sensitivity of the assay.

3.2.5 Optimisation of derivatisation

The development and optimisation process began with underivatised steroids. However, it became apparent midway through the method development process that without derivatisation, the method was not sensitive enough to detect analytes in a physiologically relevant manner (target LOQ was approximately 10 pg on column). The lack of sensitivity of the underivatised method was probably contributed by poor ionisation of neutral steroids with electrospray ionisation (ESI), which was the ionisation mode that was available for our instrument in the facility. Thus, a screening for the most suitable derivatisation agent had to be carried out. Five derivatisation agents were chosen based on a literature review, and advice provided by Prof Ruth Andrew and Dr Shazia Khan from the Mass Spectometry Core Facility at the Centre for Cardiovascular Science within the University of Edinburgh.

2 µg of each steroid standard was individually prepared from the 1 mg/mL stock and dried in the vacuum concentrator (SpeedVac), following which derivatisation agents were added according the conditions listed in Table 3.2. Derivatised steroids then underwent the same optimisation procedures as the underivatised steroids, first with MS optimisation via syringe infusion (as per section 3.2.3). Due to the nature of the

derivatisation reagents, PTSI-derivatised steroids were analysed in negative ESI mode while the rest of the derivatised steroids were analysed in positive ESI mode. After determining the optimal fragmentation and cut-off parameters, LC optimisation was then carried out (as per section 3.2.4), using the same choice of column and mobile phase optimised for underivatised steroids. A combined preliminary LC-MS method with multiple reaction monitoring was generated if the method proved to be promising.

As Girard's T showed the most potential, derivatisation conditions of Girard's T were further optimised. Incubation time and three types of acid catalyst (formic acid, acetic acid, trifluoroacetic acid) in different concentrations (0.1%, 0.2%, 0.5%, 1%) were tested, and the AUC were compared. The conditions that gave the largest AUC for extracted ion chromatograms (EIC) in the MS2 mode (i.e. incubation at 37^{o}C for 30 min with 0.2 % formic acid) were subsequently used as the Girard's T derivatisation protocol accompanying sample processing optimisation.

Derivatisation agent	**Derivatisation conditions**	**Functional group**	**Reference**
DMABC 4-Dimethylamino benzoyl chloride	Add 400 µL of 1mg/mL DMABC in acetone, incubate at 60^{o}C for 10 min.	Aliphatic hydroxyl (-OH)	Sigma technical note
HTP 2-hydrazino-4-(trifluoromethyl)-pyrimidine	First, resuspend in 50 µL acetonitrile. Then add 50 µL of 0.2 mg/mL HTP in acetonitrile with 0.05% TFA. Incubate at 60^{o}C for 1 hr.	Carbonyl (=O)	(Weng et al., 2010)
PTSI p-toluenesulfonyl isocyanate	First, resuspend in 100 µL acetonitrile. Then add 20 µL of 10% PTSI, vortex in room temperature for 2 min. Quench reaction with 20 µL of water.	Aliphatic hydroxyl (-OH)	(Ahonen et al., 2013)

Dansyl chloride (DnCl) *Protocol 1*	Add 100 µL of 100mM $NaHCO_3$ (pH 10) to sample, then 100 µL of 1mg/mL DnCl in acetone. Incubate 60^{o}C for 10 min.	Phenolic hydroxyl	(Wang et al., 2013)
Dansyl chloride (DnCl) *Protocol 2*	First, add 100 µL of 2% (v/v) N,N-diisopropylethylamine in CH_2Cl_2 to sample, followed by 100 µL of a solution of 10 mg/mL DnCl and 10 mg/mL DMAP in CH_2Cl_2, incubate at 60^{o}C for 1hr.	Aliphatic hydroxyl (-OH)	(Tang and Guengerich, 2010)
Girard's T reagent	Add 400 µL of 1 mg/mL of Girard's T reagent, in methanol with 0.2%FA, incubate at 37^{o}C for 30 min. Stop reaction with the addition of 50 µL of 5% ammonium hydroxide in methanol.	Carbonyl (=O)	(Dury et al., 2016)

Table 3.2: Derivatisation agents utilised in this study. Derivatisation conditions were taken from the respective references and modified for our method. Abbreviations: TFA: Trifluoroacetic acid, DMAP: 4-(dimethylaino)-pyridine, FA: Formic acid.

3.2.6 Sample processing optimisation

3.2.6.1 Samples used for sample processing optimisation

The LC-MS and derivatisation optimisation in the previous section was carried out using steroid standards in neat solvent (methanol). Sample processing optimisation however, needed to be carried out in samples that would closely mimic the biological tissues of interest (in our case, rat plasma and brain).

Standards in surrogate matrix: 4% (w/v) bovine serum albumin (BSA; VWR, Leicester, UK) in PBS (pH 7.4) was selected as the surrogate matrix, due to its similarity in albumin content to plasma, and can therefore form conjugates with steroids (van de Merbel, 2008). To reduce endogenous steroid contamination from BSA, 4% BSA was treated with 2% (w/v) activated charcoal (Sigma, UK) on a roller at 4^{o}C overnight and then filtered with 0.22 µm filters (Millipore) before being used as a surrogate matrix for standard calibrants.

Brain samples: A female rat brainstem was homogenised in methanol and was used during the initial stages of sample clean-up optimisation. The homogenate was split equally into various tubes, and each tube was spiked with 0.5 µg of steroids so as to compensate for the low amounts of some endogenous steroids present (e.g. testosterone).

3.2.6.2 Tissue homogenisation and extraction

A sample extraction and homogenisation protocol was devised based on a combination of various different studies in our literature review (Caruso et al., 2010, Higashi et al., 2007, Rustichelli et al., 2013). For most studies, homogenisation was carried out in acidified methanol or acetonitrile (with either formic acid or acetic acid). The organic solvent ensures the solubility of the steroids, whilst acid aids protein precipitation, as well as the disruption of protein-steroid complexes so that total amount of steroids can be measured (Makin, 2010). 500 µL of methanol with 1% FA was selected as the sample extraction solvent, based on previous studies, and partly due to its similarity with the mobile phase B in the LC-MS method (Rustichelli et al., 2013). Physical homogenisation was carried out with a handheld homogeniser (KIMBLE, VWR Ltd, Leicestershire, UK), with removable polypropene pestles that were replaced for each sample, so that numerous brain samples could be processed in a batch, increasing throughput. Samples were then briefly sonicated on ice, a procedure that was also observed in many protocols, which aids in further breaking up the tissue and cell membranes, releasing steroids from the cytosol. The homogenates were then incubated on dry ice for 30 min to aid protein precipitation. Following which, the samples were centrifuged and re-extracted again (with another 500 µL of methanol/1% FA) to ensure thorough extraction.

3.2.6.3 Sample clean-up

Two commercially available sample clean-up methods were selected for comparison, (i) a phospholipid removal cartridge (Supelco Hybrid SPE Phospholipid Cartridge, Sigma, #55261-U, bed weight 30 mg) and (ii) a C18 solid phase extraction cartridge (Supelco Discovery DSC-18 SPE Cartridge, Sigma, #52602-U, bed weight 100 mg). Given the abundance of phospholipids in cell membranes, the presence of phospholipids can be a major contribution to matrix effects. The Hybrid SPE cartridge removes phospholipids by interaction with zirconia ions present on the sorbent (Bylda et al., 2014). C18 SPE, on the other hand, works through

hydrophobic exchange, where analytes and contaminants are first retained on the sorbent on the basis of hydrophobicity, and are differentially eluted depending on the elution solvent. The Hybrid SPE and C18 SPE methods were compared using rat brainstem samples spiked with steroid standards, which is rich in white matter and will have largest amounts of contaminating lipids, as well as using steroid standards in 4% BSA.

The two methods were compared based on two parameters, (i) the intensity of "background" signals, and (ii) signal intensity of the analytes of interest. "Background" signals are assessed by comparing total ion chromatogram (TIC) peaks in MS1 mode. The intensity of TIC peaks represents the total number of ions passing through the detector at any one point, and given the complex nature of biological matrices, are likely to represent contaminants. The method that generates TIC peaks of lower intensity is preferred. Signal intensity of the analytes were compared by assessing extracted ion chromatograms (EIC) in fragmentation mode (MS2). The method that generates greater EIC peaks representing analytes of interest is preferred.

In Hybrid SPE and C18 SPE sample clean-up, the first step of the procedure is always conditioning, usually with methanol, which activates the ligands on the surface of the sorbent. This is followed by equilibration, using the solvent that the analyte is dissolved in, in order to maximise the analyte-sorbent interaction in the subsequent steps, which then differ for Hybrid SPE and C18 SPE (Fig 3.8).

For Hybrid-SPE cartridges, following conditioning (1 mL of methanol) and equilibration (0.5 mL of methanol/1%FA), samples (in 900 µL of methanol/1%FA) were then loaded through the column, and flow through was collected in a fresh 1.5mL Eppendorf tube. As the sorbent traps phospholipids, and the steroids of interest are in the flow-through fraction. An additional 400 µL was loaded as wash, and flow-through was also collected in the same tube.

For C18 SPE, the process is more laborious, as it works via hydrophobic exchange, where steroids are first trapped in the sorbent, and then eluted in the subsequent steps. The analyte is first suspended in solvent with a lower organic composition (e.g. 30% methanol), and then loaded into the cartridge, where they will adsorb on the sorbent. The washing step, at a higher percentage of methanol (e.g. 40 or 50%

methanol), will remove a portion of these contaminants. Elution, at an even higher percentage of methanol (e.g. 85%) will elute target steroid analytes, whilst the residual highly hydrophobic contaminants remain trapped in the sorbent.

To ensure that none of the analytes of interest are lost in the process, a separate test with increasing amounts of methanol was first carried out to determine the fractions in which analytes will elute. A combined steroid standard was first diluted in a 10% methanol solution. Methanol concentration was then sequentially increased by 10% each time, and the flow-through was collected, dried, derivatised and analysed on the LC-MS. This trial experiment showed that most steroids are eluted in the 50% to 80% methanol fraction. Based on the results, as well as several other studies, a wash of 50% methanol was chosen for analyses where 11-DHC was not included in the detection, while 40% methanol was chosen as a wash for analyses where 11-DHC was included in the measurements. An elution step using 85% methanol was chosen for all cases.

In both cases, the collected eluate was then dried in a vacuum concentrator (Savant SpeedVac, ThermoFisher) overnight, derivatised using Girard's T reagent as per the conditions outlined in section 3.2.5, then analysed on the LC-MS as per the conditions outlined in section 3.2.3 and 3.2.4.

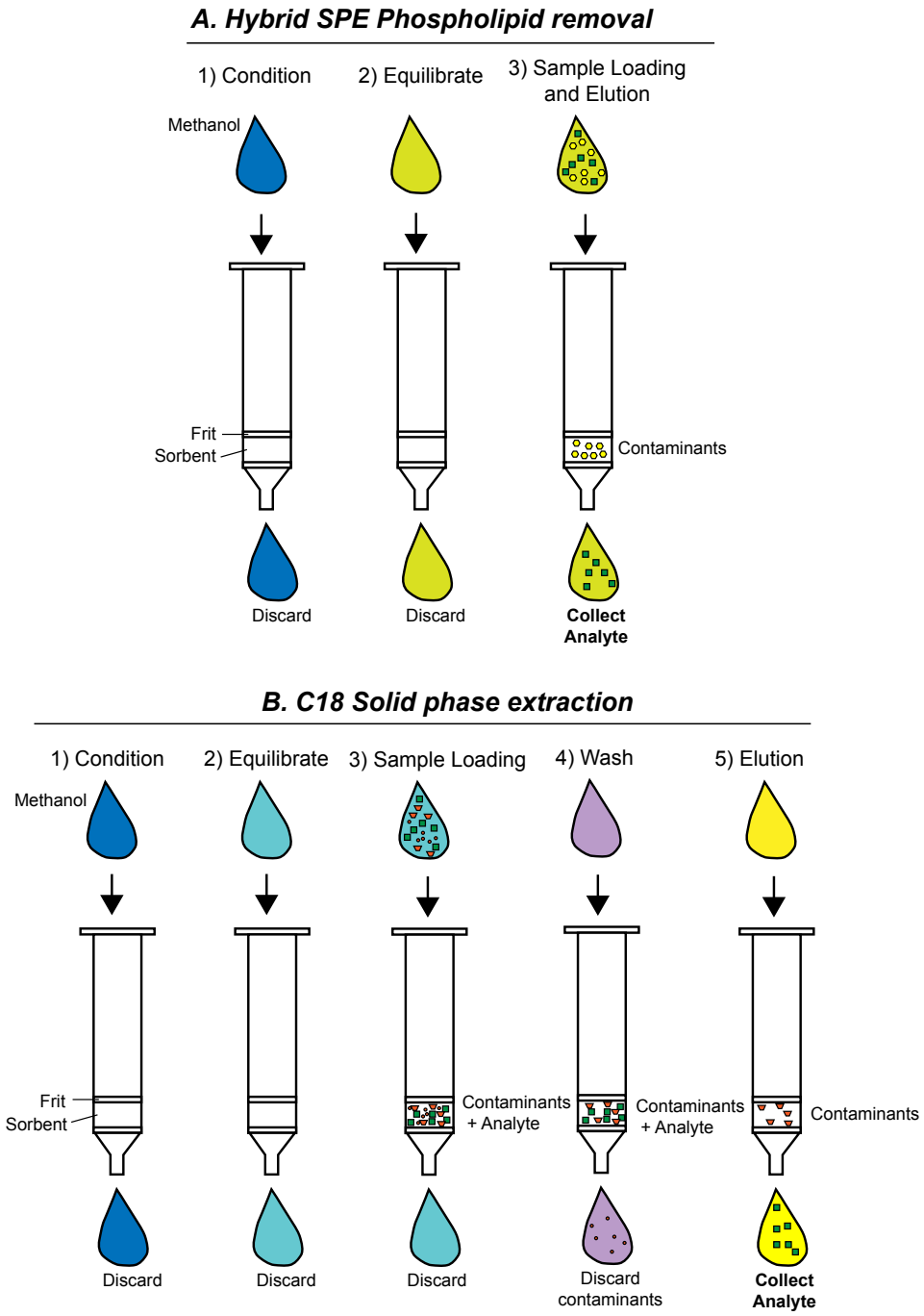


Figure 3.8: Comparison of two sample clean-up methods, phospholipid removal (Hybrid SPE) and C18 solid phase extraction (C18 SPE).

3.2.7 Assay validation parameters

3.2.7.1 Standard calibrants and internal standards

A combined sample containing 10 µg/mL of each steroid standard in methanol was diluted 40-fold in the surrogate matrix, 4% BSA, into a 250 ng/mL solution. A seven-point calibration curve was generated by diluting 25 ng/mL standards 2.5-fold, generating calibration points of 10 ng/mL, 4 ng/mL, 1.6 ng/mL, 640 pg/ml, 256 pg/mL and 102.4 pg/ml. 100 µL of standard calibrants were used for sample processing. A BSA only blank sample was prepared with each standard curve as a negative control, but was not used in the calibration.

Deuterated internal standards (ISTDs) consisting of progesterone-d9, corticosterone-d4 and allopregnanolone-d5 were combined into a 10 µg/mL solution in methanol. They were diluted 400-fold into a 25 ng/ml solution consisting of 50% methanol/ 50% PBS. 20 µL of the ISTD mix was added to each sample, including the BSA only blank sample.

3.2.7.2 Assessment of linearity

Calibration curves were constructed using the ratio of peak area of the target analyte to the peak area of the ISTD using regression analysis in the QuantAnalysis software. Coefficients of determinant (r^2) indicate how well the regression line fits the data, and the calibration curves are acceptable if values were >0.99. Weightings of 1, 1/x, and $1/x^2$, with and without forcing the intercept through zero, were compared.

3.2.7.3 Limit of quantification (LOQ) and limit of detection (LOD)

The lower limit of quantification (LOQ) was defined as the lowest standard calibrant with a peak that is identifiable and discrete with a relative standard deviation of less than 20% and at least five times the signal of the blank. The lower limit of detection (LOD) is defined as the lowest amount of analyte necessary to obtain a signal that can be distinguished from the background noise (signal to noise ratio >5). Only the LOQ was investigated in this assay validation procedure.

3.2.7.4 Recovery

Recovery was assessed by comparing the signal intensity of standards spiked before and after C18 solid phase extraction (n=3), using 4% BSA as sample matrix.

Recovery was investigated at three concentration levels, where low (50 pg), mid (200 pg) or high (800 pg) amounts of steroids were added into the sample.

Recovery (%) was calculated as:

$$\frac{\text{Peak area of pre SPE-spiked sample}}{\text{Peak area of post SPE-spiked sample}} \times 100\%$$

3.2.7.5 QC standards

Assessment of accuracy and precision was carried out using quality control (QC) samples consisting of steroid standards prepared in 4% BSA, in low (500 pg/ml), mid (2000 pg/ml) or high (8000 pg/ml) concentrations. 100 µL of each QC sample was used for sample processing, and 20 µL of ISTD was added as per standard calibrants. QC standards were processed alongside a set of standard calibrants, and the values obtained were used to determine accuracy and variability.

3.2.7.6 Accuracy

Accuracy refers to how closely the values reflect the actual concentration of steroids in the sample. Accuracy was calculated from the means of three independent samples for each concentration level using the following formula:

$$\frac{\text{Obtained concentration}}{\text{Known concentration}} \times 100\%$$

3.2.7.7 Variability

Variability is presented as % coefficient of variation (%CV), calculated with the following formula:

$$\%\text{CV} = \frac{\text{Standard deviation of samples}}{\text{Mean of samples}} \times 100\%$$

Four forms of variability are investigated here, which contribute to the precision of the assay.

Intra-assay variability refers to the %CV of the values obtained from three independent samples measured in duplicate in a single run, against a single calibration curve. Intra-assay variability assesses the consistency of sample preparation, the stability of the analyte and fluctuations in performance of the LC-MS.

Inter-assay variability refers to the %CV of mean measurements across three different runs, against three different calibration curves. Inter-assay variability accounts for the variation in sample and standard curve preparation on different days. Apart from the fluctuations in instrumental performance, the consistency of the slope of the standard curve on different days, as well as possible variations in the preparation of different solvent batches are taken into account.

Injection-to-injection variability is calculated from the mean %CV value of the duplicate injections. This accounts mainly for fluctuations in LC-MS performance within a single run, which can include the fidelity of injection volume, carry-over, ionisation and fragmentation efficiencies and column performance.

Total variability refers to the %CV of all the single injections obtained across various runs, irrespective of the day samples were run. This accounts for whether the intra-assay, inter-assay and injection-to-injection variability may have a cumulative effect. The total variability is calculated using the %CV formula mentioned above, where standard deviation of values obtained from all single injections across all days (n=15) was divided by the the mean of all single injections across all days (n=15).

3.2.8 Biological validation of the method in rat plasma and brain tissues

The LC-MS method was biologically validated on rat plasma and brain tissues collected from control and swim-stressed adult rats. The study has been published in Sze et al. (2018) and is attached in Appendix B. At the time of publishing, the LC-MS method could only reliably detect 8 steroids (corticosterone, DOC, DHDOC, progesterone, DHP, allopregnanolone, pregnenolone, testosterone), and only used only progesterone-d9 as internal standard. The publication (Sze et al., 2018; Appendix B) contains both a validation assay for these 8 steroids, as well as results of steroid concentration measurement from the stressed and non-stressed rat plasma and brain tissues. As the method used in the publication (Sze et al., 2018;

Appendix B) differs with the slightly modified method used in the rest of the thesis, it is referred to as the "initial published method" in this chapter.

3.2.8.1 Animals and acute stress paradigm (forced swimming)

Briefly, male and female Sprague-Dawley rats (n=7 per group per sex) were bred in-house and underwent forced swimming for 2 minutes when they were 21 weeks old. Stressed rats were returned to their home cage after the swimming bout. Thirty minutes after the onset of swimming stress, rats were killed by conscious decapitation. For the control group, rats (n = 7 per group per sex) remained undisturbed in their home cage prior to killing. Upon decapitation, trunk blood was collected and brains were rapidly removed. The regions of interest (frontal cortex, hypothalamus, amygdala, hippocampus, brainstem) were dissected and frozen on dry ice. All experiments and tissue collection were performed in male and female (randomly cycling) rats between 10.00 am and 12.30 pm at the Roslin Institute, carried out by Mrs Helen Cameron and Dr Yu-Ting Lai.

Forced swimming was chosen as a stressor because firstly, it is a robust activator of the HPA axis, and has been shown to rapidly induce an increase in concentrations of corticosterone, pregnenolone and allopregnanolone in the brain (Purdy et al., 1991, Vallée et al, 2000), therefore is likely to bring out differences between treatment groups. This time-point of killing and collecting of samples (30 minutes) has also previously been shown to correspond with a peak in stress-induced corticosterone and allopregnanolone concentrations in the brain following swim stress (Cullinan et al., 1995). Secondly, forced swimming is a combined physical and psychological stressor, therefore activates stress-responsive nuclei in both the forebrain and hindbrain (Cullinan et al., 1995), and various brain regions would be affected by the manipulation.

3.2.8.2 Sample processing and initial published LC-MS method

Samples from all four groups from any given region were processed in the same run, together with standard calibrants (prepared as per section 2.5.1). Brain samples were processed as per the methods mentioned in section 2.5.2, where plasma and brain regions were weighed, homogenised in methanol/1% formic acid and underwent C18 solid phase extraction. Samples then underwent Girard's T

derivatisation (as per section 2.5.2) before being subjected to LC-MS analysis, which are detailed in Appendix B. A validation assay using QC standards was also included (Appendix B, Supplementary Info), and the assessment of assay performance was based on calculations detailed in section 3.2.7.

3.2.8.3 Differences between the initial published method and the method used in this thesis

The method used in this thesis (in Chapter 4, 5 and 6) and assay validation (from section 3.3.4) reflects an improved method from this initial published assay (Sze et al., 2018; Appendix B), due to an upgrade of the instrumentation (to a refrigerated autosampler), as well as the addition of two more additional internal standards, corticosterone-d4 and allopregnanolone-d5. THDOC was also not included in this initial published assay due to its poor validation characteristics (calibration curve was not linear).

3.3 RESULTS

3.3.1 Results of screening for different derivatisation agents

Table 3.3 compares the AUC for extracted ion chromatograms (EIC), detected at MS1 mode for underivatised steroids and steroids derivatised with the five derivatisation agents detailed in Table 3.2. In MS1 mode, EICs represent the intensity of the precursor ion without fragmentation.

Underivatised steroids could be detected in MS1 mode, however, fragmentation was poor for all the underivatised steroids in MS2 mode, leading to overall poor sensitivity. The LOD of the underivatised steroids using that method was >10 ng/ml for most steroids in our pilot study, which was insufficient given the much lower physiological concentrations of steroids in the brain (a target LOQ of approximately 100 pg/ml was set).

DMABC derivatisation, which targets hydroxyl groups, showed potential, resulting in the largest number of steroids being quantified. 3α-diol and 3β-diol, which has never been detected in any published studies, could be detected with DMABC derivatisation. However, the derivatisation agent was sensitive to both moisture and air, and the reaction yield became significantly lower as time progressed, possibly due to the degradation of the reagent. The development of a method with DMABC derivatisation was thus abandoned. Additionally, as allopregnanolone was a focus in our studies, it was important to also quantify progesterone and DHP, the precursors of allopregnanolone. The focus was shifted then to finding a derivatisation agent that could target carbonyl groups (which progesterone, DHP and allopregnanolone all possess).

Girard's T derivatisation and HTP derivatisation, targeting carbonyl groups, both proved to be robust derivatisation agents at the early stages of optimisation. However, HTP showed slightly lower EIC signal in MS2 for most analytes. Higher temperature was also needed for the derivatisation reaction, which was less ideal. Girard's T was thus chosen as the preferred derivatisation agent in this study.

Analyte	Area under curve for EICs in MS1 mode					
	None	DMABC	DnCl	PTSI	HTP	Girard's T
Corticosterone	1.8×10^9	1.4×10^9	n.d.	n.d.	1.1×10^9	2.4×10^9
DOC	1.8×10^9	2.3×10^9	3.8×10^8	2.1×10^7	1.6×10^9	2.2×10^9
DHDOC	3.8×10^8	2.1×10^8	n.d.	3.6×10^7	1.5×10^8	1.6×10^8
THDOC	1.5×10^8	1.7×10^9	3.0×10^8	2.8×10^7	1.4×10^6	2.9×10^8
Progesterone	2.8×10^9	n.d.	n.d.	n.d.	1.6×10^9	2.6×10^9
DHP	7.2×10^8	n.d.	n.d.	n.d.	3.5×10^8	9.2×10^8
Allopregnanolone	3.7×10^8	1.5×10^9	n.d.	3.1×10^7	2.5×10^8	1.3×10^9
Pregnenolone	3.4×10^8	1.1×10^9	n.d.	1.1×10^7	3.9×10^8	8.8×10^8
Oestradiol	n.d.	2.1×10^9	6.4×10^8	1.1×10^7	n.d.	n.d.
Testosterone	1.5×10^9	3.8×10^9	1.7×10^9	1.8×10^7	2.9×10^9	8.7×10^8
DHT	5.6×10^8	2.0×10^9	6.8×10^8	1.8×10^7	1.0×10^8	3.2×10^7
3α-diol	n.d.	7.0×10^8	6.0×10^8	5.1×10^7	n.d.	n.d.
3β-diol	n.d.	4.2×10^8	2.3×10^8	1.5×10^7	n.d.	n.d.

Table 3.3: Area under curve for extracted ion chromatograms for precursor ions in MS1 only mode. None: No derivatisation, DMABC: 4-Dimethylamino benzoyl chloride, DnCl: Dansyl chloride, PTSI: p-toluenesulfonyl isocyanate, HTP: 2-hydrazino-4-(trifluoromethyl)-pyrimidine. For derivatisation conditions see Table 3.2.

3.3.2 Finalised MS and LC parameters development based on Girard's T derivatisation

The finalised LC-MS method based on Girard's T derivatisation was able to detect and quantify 10 steroids in a single injection. The representative MS2 chromatogram is shown in Fig 3.9, where the peaks were well-resolved and had good shapes. The LOQ of this method was 102.4 pg/ml for all analytes, whilst its accuracy and precision based on QC standards is presented in the assay validation section (section 3.3.4). This LC-MS method was also used for the optimisation of sample processing (section 3.3.3).

The 10 steroids that were quantified are corticosterone, 11-DHC, corticosterone, DOC, DHDOC, THDOC, progesterone, DHP, allopregnanolone, pregnanolone and testosterone. Three internal standards were used in this method, corticosterone-d4, progesterone-d9 and allopregnanolone-d5. A total run time of 19 min was required

to detect these 10 steroids (Fig 3.10A). In addition, a condensed gradient profile with a shorter run time of 10 min was developed for the detection of corticosterone and 11-DHC only, which will be relevant in Chapter 5 (Fig 3.10B).

The transitions monitored for MRM and optimised fragmentation parameters are listed in Table 3.4. The m/z of the all the precursor ions and fragment ions corresponded to the chemistry of the Girard's T reaction and the fragmentation patterns of the Girard's T hydrazone (Fig 3.11). Mass spectral peaks showing fragmentation patterns of each analyte in MS2 mode are shown in Fig 3.12.

Even though DHT contains a carbonyl group and could be detected in MS1 mode during the MS optimisation stage (Table 3.3), albeit with a lower intensity, it had poorer signals for MS2 and could not be detected at all during LC optimisation stage. It was thus not included in the final LC-MS method.

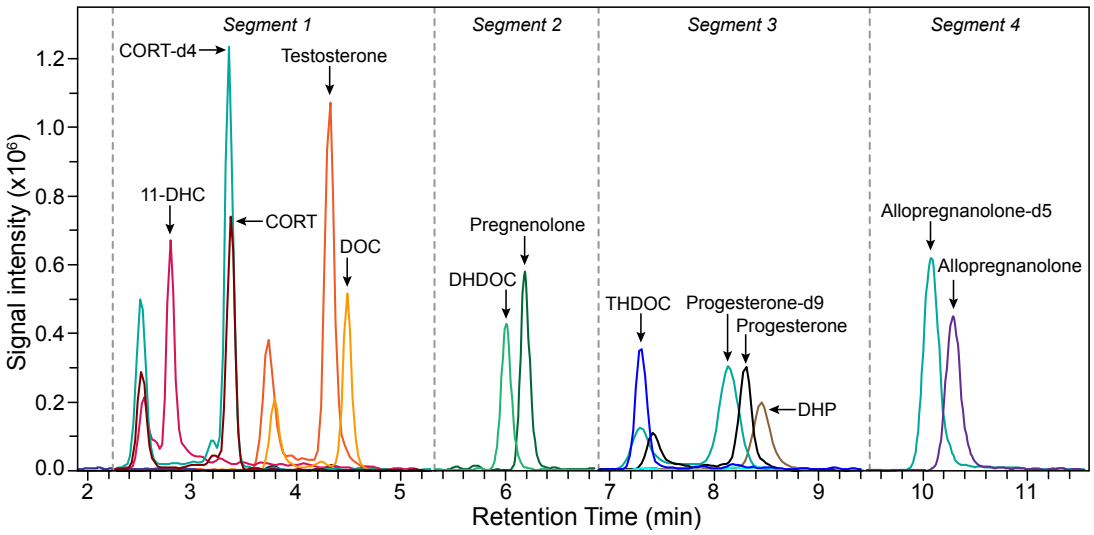


Figure 3.9: Extracted ion chromatograms of 4 ng/ml standards in 4% BSA. Girard's T derivatisation resulted in double peaks for some analytes, possibly due to the *syn* and *anti*-configurations of the derivatisation agent. The major peak (always the peak that had a later retention time) was used for quantification and the ratio between the minor and major peak was always the same.

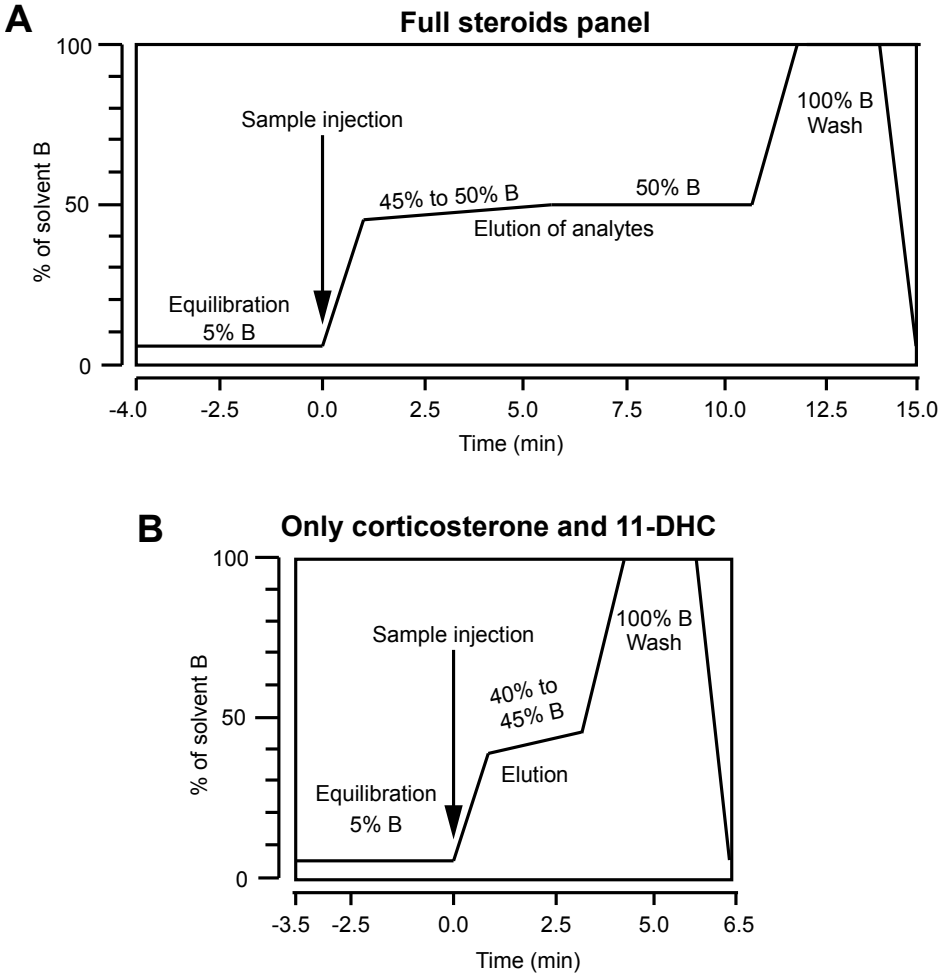


Figure 3.10: Gradient characteristics. Two gradient profiles were used in this thesis, one for the quantification of an entire panel of steroids (A), and the other an abbreviated protocol for the quantification of only corticosterone and 11-DHC (B).

In (A) Full steroids panel: Total run time of 19 min. -4.0 min to 0 min: 5% B, 0 min to 1.0 min: 0% B to 45% B, 1.0 min to 6.0 min: 45% B to 50% B, 6.0 min to 11.0 min: 50% B, 11.0 min to 12.0 min: 50% to 100% B, 12.0 min to 14.0 min, 100% B (wash), 14.0 min – 15.0 min 100% B to 5% B.

In (B) Corticosterone and 11-DHC only panel: Total run time of 10 min. -3.5 min to 0 min: 5% B, 0 min to 1.0 min: 0% B to 40% B, 1.0 min to 3.0 min: 40% B to 45% B, 3.0 min to 4.0 min: 45% B to 100%B, 4 min to 6.0 min: 45% to 100% B, 6 min to 6.5 min 100% B to 5% B.

Analyte	Molecular weight	Precursor ion (m/z)	Fragment ion (m/z)	Retention time (min)	Segment	Amplitude; Cut-Off
11-DHC	344.5	458.2	399.2	2.8	1	117; 0.80
Corticosterone	346.5	460.2	401.1	3.5	1	117; 0.80
Corticosterone-d4	350.5	464.3	405.1	3.5	1	117; 0.80
Testosterone	288.4	402.2	343.1	4.4	1	117; 0.80
DOC	330.5	444.2	385.1	4.6	1	120; 0.95
DHDOC	332.5	446.2	387.1	6.1	2	116; 0.80
Pregnenolone	316.5	430.2	371.1	6.3	2	116; 0.75
THDOC	334.5	448.2	389.1	8.0	3	116; 0.55
Progesterone-d9	323.5	437.5	368.3	8.2	3	116; 0.70
Progesterone	314.5	428.2	369.1	8.4	3	116; 0.75
DHP	316.5	430.2	371.1	8.6	3	116; 0.75
Allopregnanolone	318.5	432.2	373.1	10.4	4	116; 0.70
Allopregnanolone -d5	323.5	437.6	368.3	10.5	4	120; 0.75

Table 3.4: Transitions monitored and optimised mass spectral settings for Girard's T derivatised analytes. Derivatisation with Girard's T reagent produced a precursor ion $[M + G.T.]^+$, resulting in an addition of 114 Da to its absolute mass (see Figure 3.11 for chemical reaction and proposed fragmentation scheme). Upon fragmentation by collision-induced fragmentation, a product ion with neutral loss of 59.1 Da (corresponding to the loss of the trimethylamine moiety) was produced, which was used for identification and quantification. Detection was carried out via multiple reaction monitoring, with no more than 5 analytes monitored for each segment. The width of detection was ± 2.0 m/z for the precursor ions, and ± 0.5 m/z for the fragment ions during data analysis.

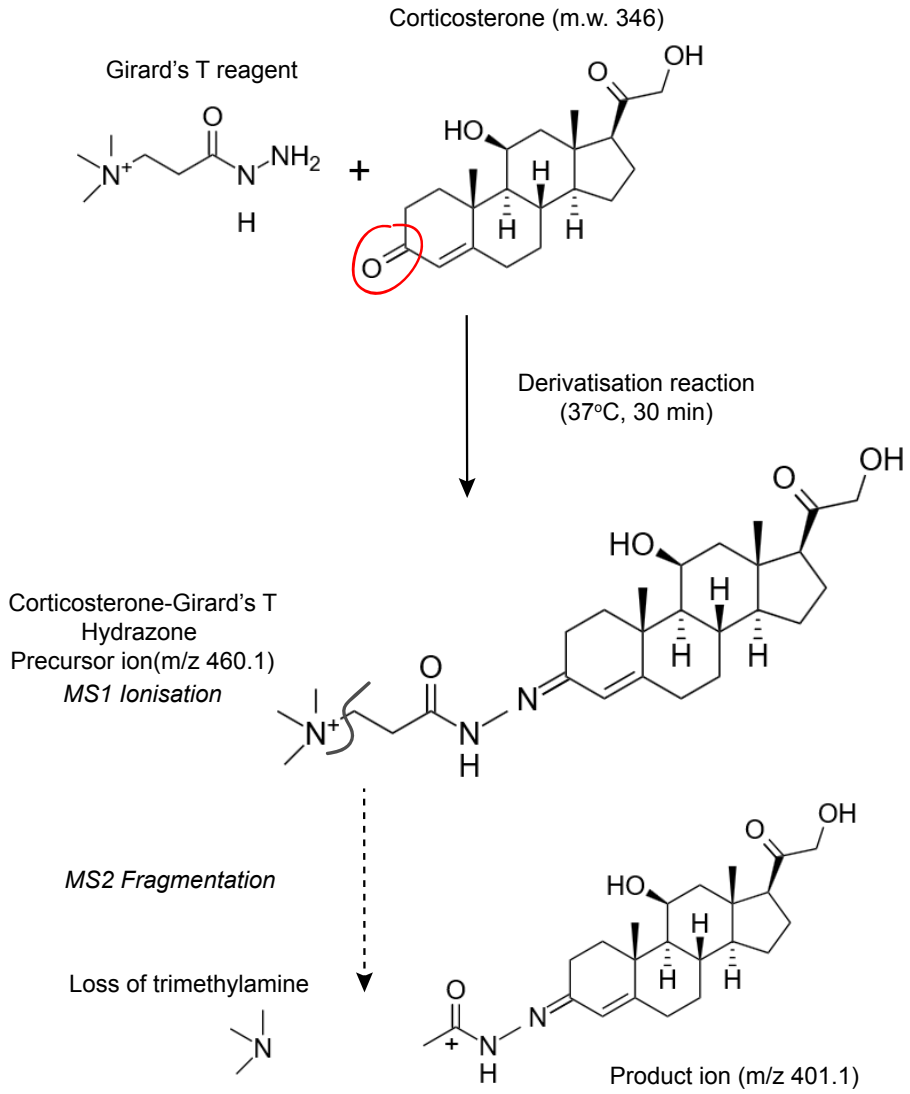


Figure 3.11: Girard's T derivatisation and proposed fragmentation scheme. The reaction of the C3 carbonyl group (red circle) of corticosterone with Girard's T reagent at 37°C for 30 min produces a Girard's T-corticosterone-hydrazone, which is detected on the MS1 mode with a m/z of 460.2, with an addition of 114 Da to its absolute mass (corticosterone has a molecular weight of 346). Upon fragmentation by CID, a product ion with neutral loss of 59.1 Da (corresponding to the loss of the trimethylamine moiety) is produced, and a product ion of m/z 401.1 is detected on MS2 mode.

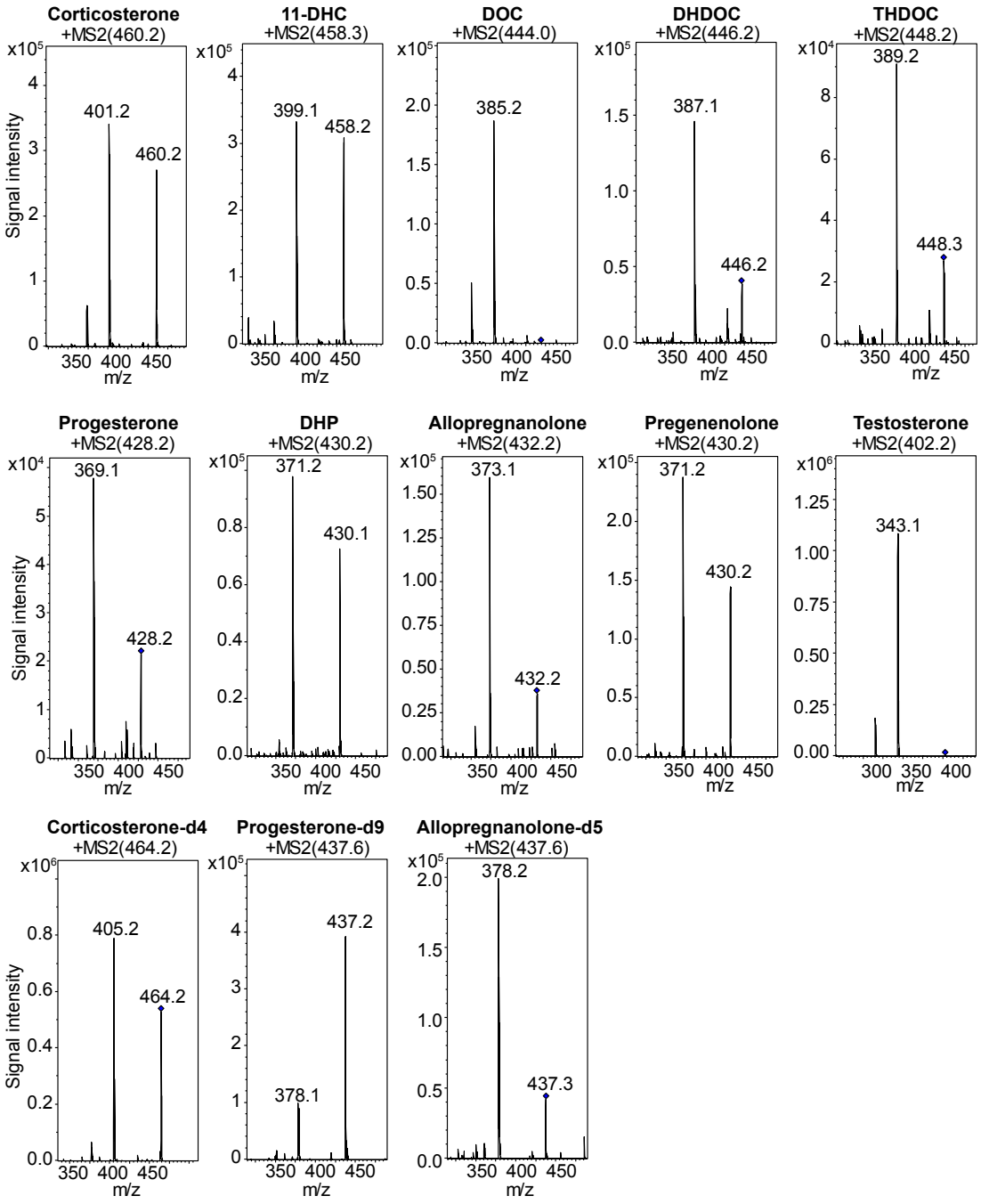
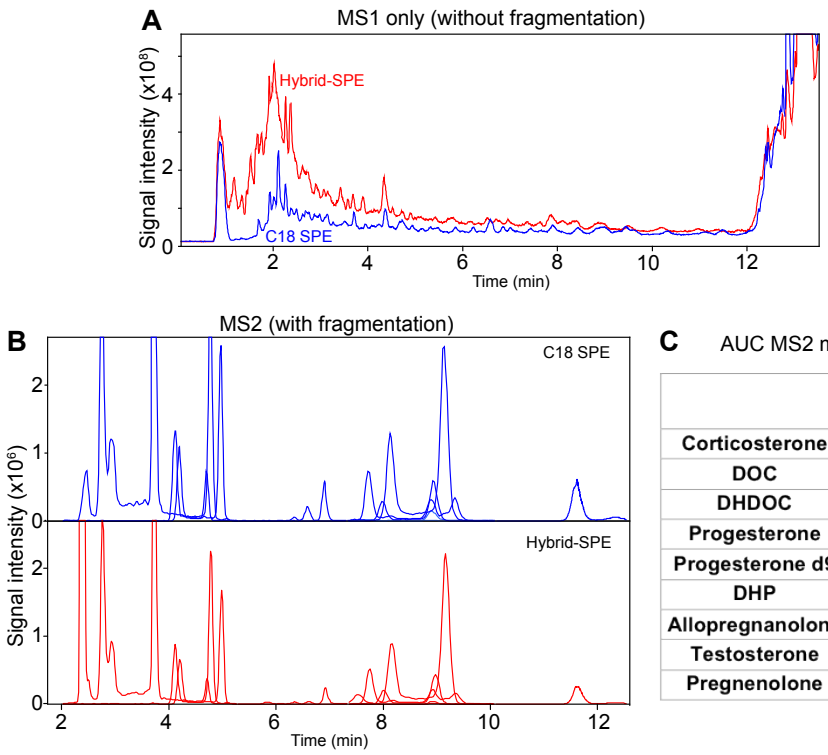


Figure 3.12: Mass spectra in MS2 mode showing relative intensities of the precursor and fragment ions. Mass spectra of the corresponding chromatographic peaks in Fig 3.9.

3.3.3 Results of sample clean-up optimisation

In MS1 mode, sample clean-up with Hybrid SPE resulted in higher TIC intensities as compared to that of C18 SPE (Fig 3.13A). As TIC quantifies the total number of ions that are detected at any one time and are likely to represent contaminants, C18 SPE is therefore a better choice due to its lower TIC intensity. In MS2 mode, the analytes of interest were isolated and fragmented. C18 SPE clean-up had slightly better responses for all analytes investigated (Fig 3.13B and C).



C AUC MS2 mode (Arbitary units)

	C18 SPE	Hybrid SPE
Corticosterone	4.1×10^7	2.6×10^7
DOC	1.1×10^7	7.1×10^6
DHDOC	1.3×10^6	2.9×10^5
Progesterone	2.5×10^7	2.0×10^7
Progesterone d9	5.5×10^6	4.3×10^6
DHP	3.0×10^6	1.9×10^6
Allopregnanolone	6.7×10^6	3.0×10^6
Testosterone	1.7×10^7	1.0×10^7
Pregnenolone	5.4×10^6	3.9×10^6

Figure 3.13: Comparison of extraction methods. (A) Sample clean-up with hybrid SPE (in red) showed greater levels of contamination as compared to that of C18 SPE (in blue), evident from the higher TIC intensity in the MS1 mode. (B) C18 SPE showed better EIC signals for almost all analytes, indicating better ionisation and fragmentation of analytes in the MS2 mode. Comparison is carried out using a rat brainstem sample spiked with 0.5 µg of steroid standards.

3.3.4 Assay performance of finalised method

3.3.4.1 Linearity of calibration curve

Calibration curves were linear between the range of 102.4 pg/ml to 25 ng/ml. Average R^2-values representing the linearity of the curve are presented in Table 3.6 and representative calibration curves are presented in Fig 3.14. Fitting of 1/X improved back-calculated accuracy of the standard calibrants. The intercept was close to y=0, however, forcing the intercept through zero for all curves improves the consistency of the method across different days (i.e. decreases inter-assay variability).

Analyte	R^2	ISTD
11-DHC	0.998	Corticosterone-d4
Corticosterone	0.998	Corticosterone-d4
DOC	0.998	Corticosterone-d4
DHDOC	0.997	Progesterone-d9
THDOC	0.994	Progesterone-d9
Progesterone	0.998	Progesterone-d9
DHP	0.998	Progesterone-d9
Pregnenolone	0.998	Allopregnanolone-d5
Allopregnanolone	0.996	Allopregnanolone-d5
Testosterone	0.997	Corticosterone-d4

Table 3.5: Summary of R^2 values which relate to the linearity of these curves, alongside the internal standards used. Standard calibration curves were all fitted to 1/X weightage and intercept set to y=0.

3.3.4.2 Limits of quantification

Limits of quantification were 102.4 ng/ml for all analytes. Representative peaks of the lowest standards (which equates to the LOQ in this case) are presented in Figure 3.15, overlaid on a BSA only blank sample. In general, peaks were of good shape and were distinct from the blank sample, and a signal-to-noise ratio was more than 5, calculated by the QuantAnalysis software.

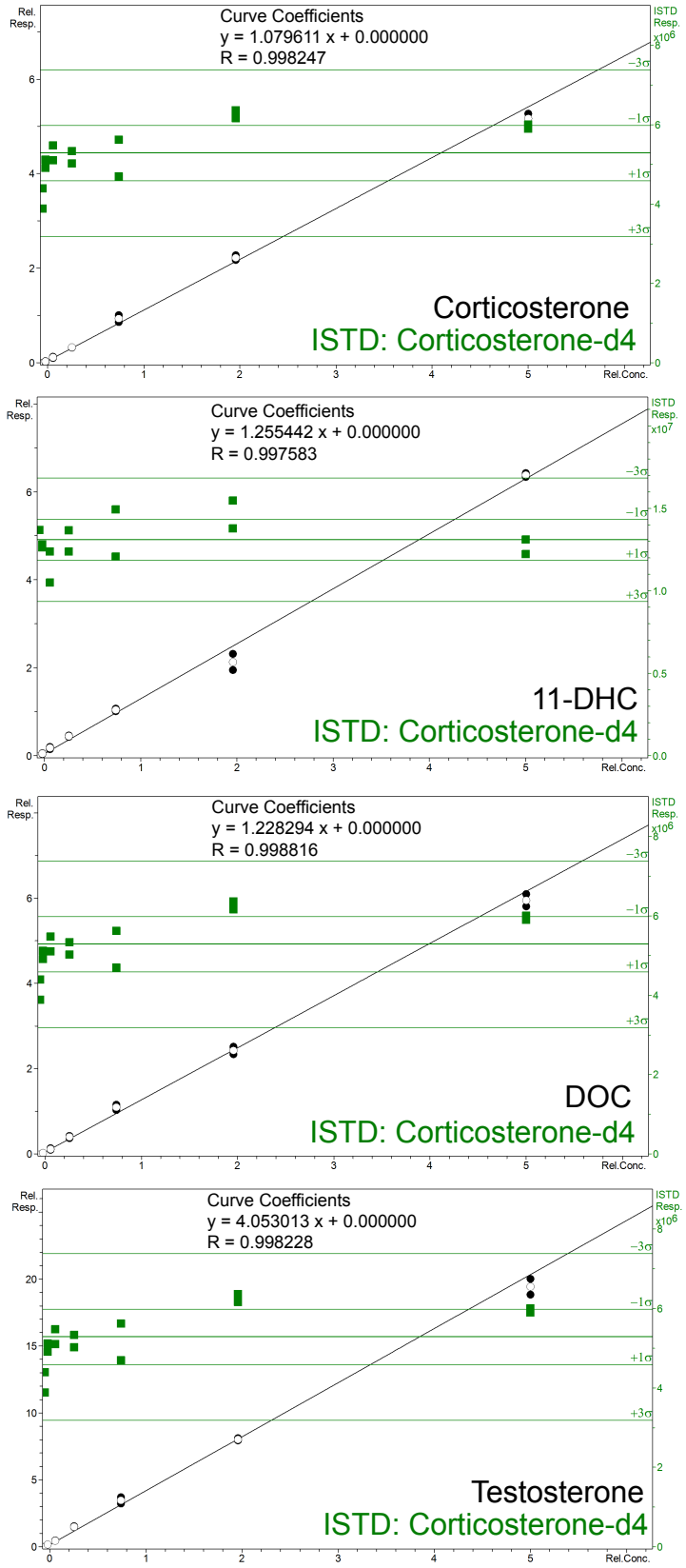


Figure 3.14A: Representative graphs of calibration curves with cortisterone-d4 as ISTD. Black circles represent each replicate of the standard calibrants, while white circles represent the mean of the two duplicates. Green squares represent the relative response of the internal standard corticosterone-d4.

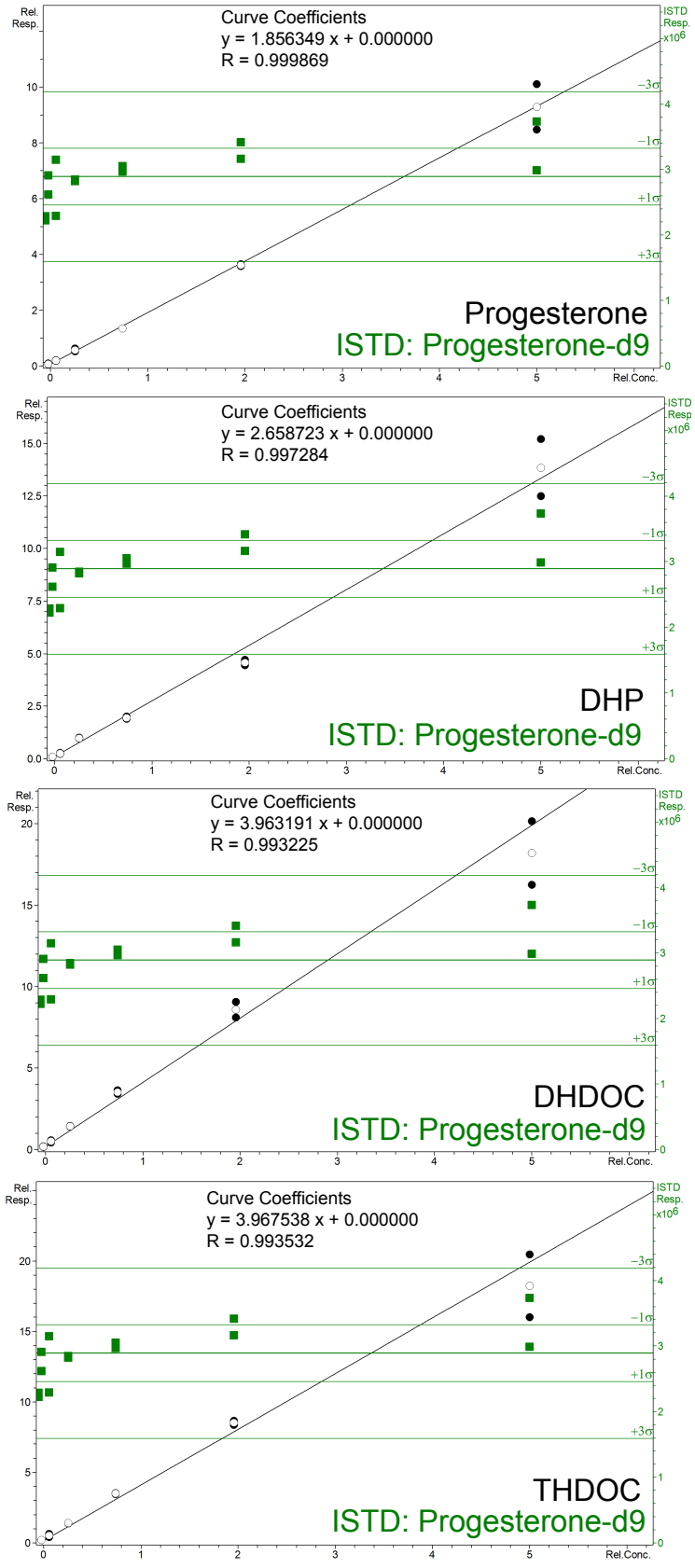


Figure 3.14B: Representative graphs of calibration curves with progesterone-d9 as ISTD. Black circles represent each replicate of the standard calibrants, while white circles represent the mean of the two duplicates. Green squares represent the relative response of the internal standard progesterone-d9.

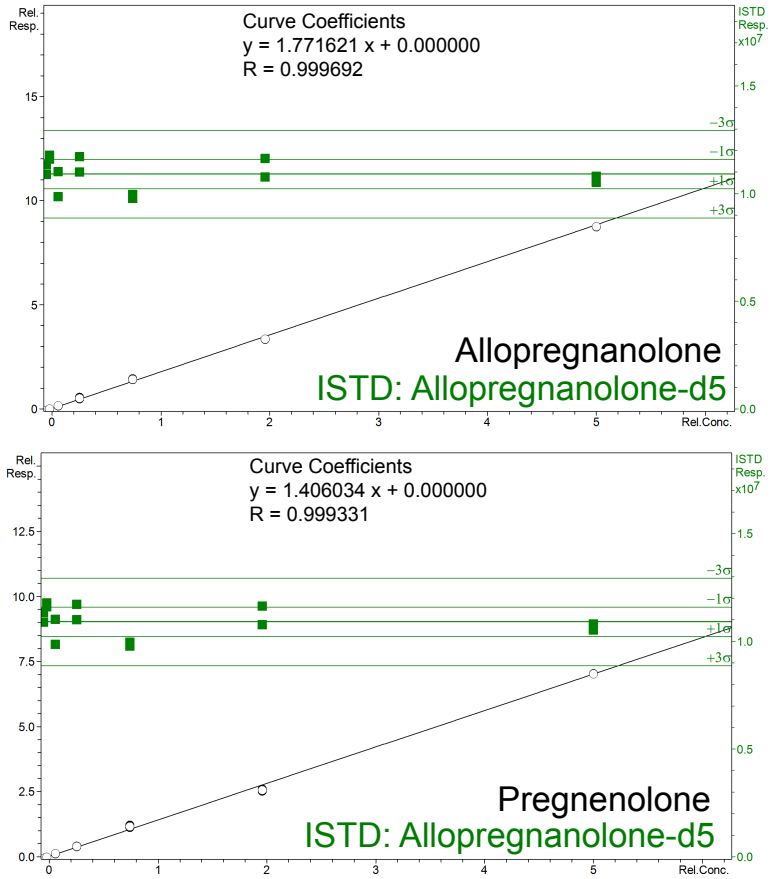


Figure 3.14C: Representative graphs of calibration curves with allopregnanolone-d5 as ISTD. Black circles represent each replicate of the standard calibrants, while white circles represent the mean of the two duplicates. Green squares represent the relative response of the internal standard allopregnanolone-d5.

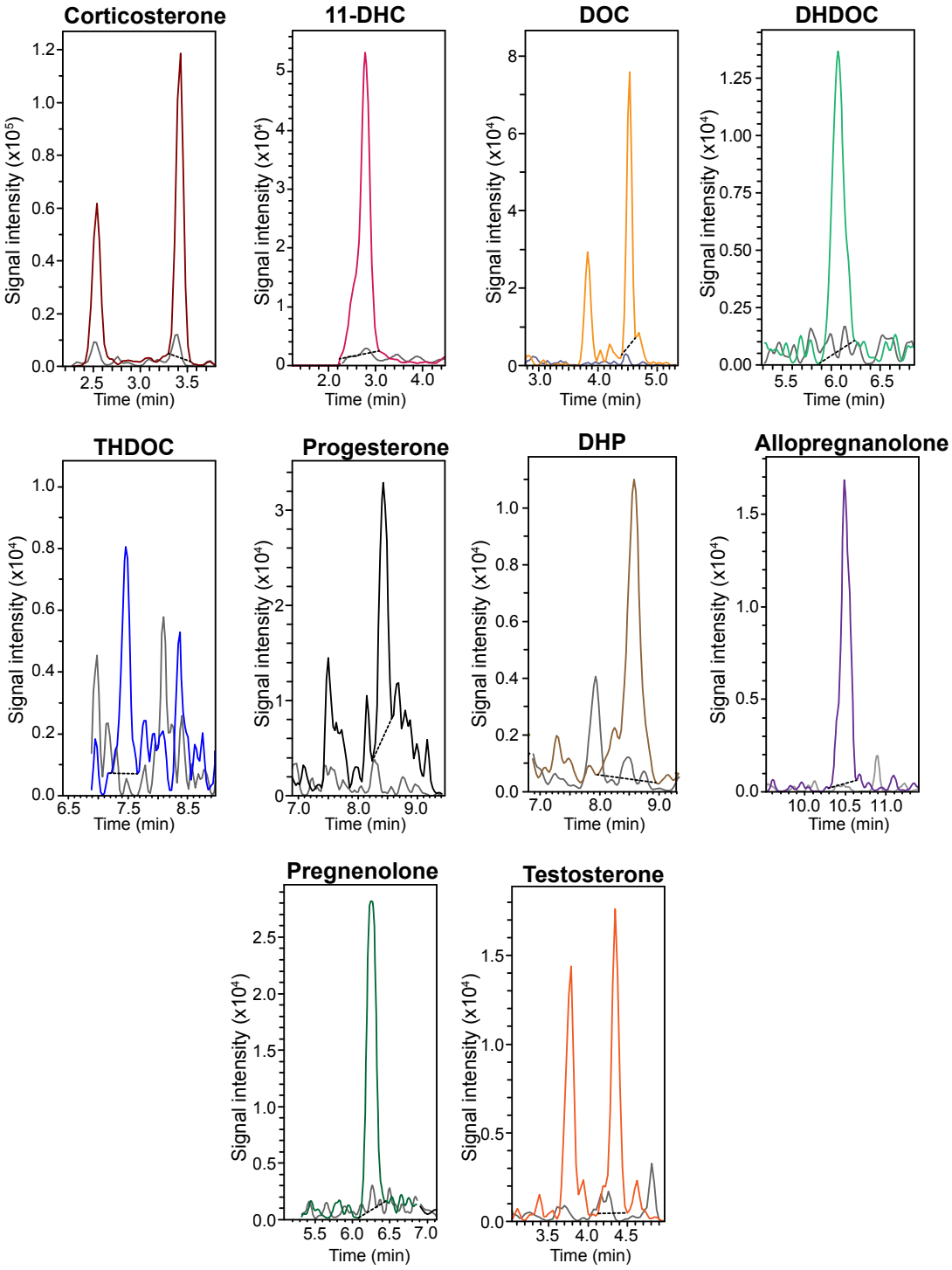


Figure 3.15: Representative peaks at limit of quantification. Peaks (coloured) at the LOQ, 102.4 ng/ml for all analytes, overlaid on a BSA only sample (grey). Signal-to-noise ratio was more than 5, as calculated by the QuantAnalysis software. The dotted line represents the line drawn for manual integration of peaks to obtain AUC. For most analytes, LOD could be lowered if standards were further diluted, while THDOC seems to have reached its LOD at this concentration.

3.3.4.3 Recovery

Recovery is summarised in Table 3.6, and ranged between 71 – 108 % for all analytes.

Analyte	QC Low	QC Mid	QC High
Corticosterone	74	71	84
DOC	78	72	92
DHDOC	95	82	108
Progesterone	71	80	83
DHP	73	70	82
Pregnenolone	88	81	95
Allopregnanolone	88	87	94
Testosterone	95	105	106

Table 3.6: Recovery of steroid analytes following C18 SPE. Recovery was investigated by spiking low (50 pg), mid (200 pg) or high (800 pg) amounts of steroid standards before and after C18 SPE into 4% BSA.

3.3.4.4 Accuracy

Accuracy is summarised in Table 3.7, and ranged between 62% - 132 % for different analytes.

	QC Low	QC Mid	QC High
Corticosterone	114	103	99
11-DHC	105	104	102
DOC	100	95	92
DHDOC	91	76	62
THDOC	95	67	63
Progesterone	125	125	108
DHP	101	98	85
Allopregnanolone	117	119	110
Pregnenolone	116	132	118
Testosterone	97	84	79

Table 3.7: Accuracy of the quantification. Accuracy was determined in low (500 pg/ml), mid (2 ng/ml) and high (8 ng/ml) concentrations of quality control (QC) standards. Accuracy was calculated as the concentration/theoretical concentration x 100%.

3.3.4.5 Variability

Analyte	QC level	Intra-assay (%CV)	Inter-assay (%CV)	Injection variation (%CV)	Total CV (%CV)
Corticosterone	QC Low	14.0	10.6	12.3	15.6
	QC Mid	9.8	5.2	3.0	8.0
	QC High	10.0	7.0	5.8	11.7
11-DHC	QC Low	7.6	12.8	9.4	10.6
	QC Mid	4.4	5.7	6.3	9.7
	QC High	8.1	5.0	7.0	9.7
DOC	QC Low	4.3	12.8	6.8	7.6
	QC Mid	3.7	5.7	3.4	4.8
	QC High	6.0	5.0	10.2	10.7
DHDOC	QC Low	0.9	14.0	9.9	17.3
	QC Mid	17.9	23.3	12.9	28.5
	QC High	10.2	24.3	6.1	16.8
THDOC	QC Low	8.9	4.4	8.5	11.5
	QC Mid	19.4	17.7	15.6	26.6
	QC High	12.3	16.7	9.9	21.1
Progesterone	QC Low	7.5	11.7	8.7	19.5
	QC Mid	14.2	6.5	13.6	16.2
	QC High	7.3	22.5	8.9	25.1
DHP	QC Low	9.0	11.2	11.4	22.1
	QC Mid	9.0	15.5	7.7	18.3
	QC High	4.2	28.0	8.1	28.2
Allopregnanolone	QC Low	2.7	10.7	4.2	11.2
	QC Mid	2.4	2.9	5.9	5.1
	QC High	5.2	5.7	5.7	7.8
Pregnenolone	QC Low	5.7	12.6	5.4	14.1
	QC Mid	3.3	13.3	5.6	14.3
	QC High	6.9	15.1	4.8	14.9
Testosterone	QC Low	4.5	18.3	7.5	13.9
	QC Mid	9.8	5.3	5.1	10.7
	QC High	2.1	11.7	7.4	11.1

Table 3.8: Intra-assay, inter-assay, injection-to-injection and total variability. Three independent samples from each concentration (QC Low, 500 pg/ml; QC Mid, 2000 pg/ml; or QC High, 8000 pg/ml) were used to assess variability, for samples that underwent SPE, derivatisation and LC-MS analysis on the same day (intra-assay) or on different days (inter-assay). Injector reproducibility assesses the variability present between duplicates. Total variability assesses variation across the different individual injections irrespective of day of injection.

3.3.5 Application of the method in rat plasma and brain tissues

An initial LC-MS method was applied to the quantification of neuroactive steroids in rat brain samples where concentrations are expected to be different, as proof-of-concept that the method can be successfully implemented in biological applications. Whilst detailed results of the measurements can be found in the published paper (Sze et al., 2018; Appendix B), several main findings are summarised here.

Firstly, the results from both validation assays, in this thesis (in section 3.3.4) and the initial published paper (Appendix B) are similar and are both within acceptable limits, indicating the consistent performance of this LC-MS method despite small modifications to the method.

Secondly, the published LC-MS method (Sze et al., 2018) was able to detect expected patterns of increase in neuroactive steroids following stress, as well as detect sex differences in hormone concentrations. It confirmed earlier reports using male rats by Purdy et al. (1991) and Vallée et al. (2000) that there is a robust increase in the concentrations of neuroactive steroids, especially pregnenolone and allopregnanolone, in the brain following acute stress. Expected sex differences in absolute steroid concentrations were also observed, where testosterone concentrations was far greater in the males in the plasma and all brain regions, while for other hormones such as pregnenolone and progesterone, the concentrations were clearly greater in the females. Moreover, when the values were compared to published data from other groups (Table 3.9), most of the values were close to these previously published. Given these observations, the LC-MS method can be considered to be biologically validated.

Lastly, apart from replicating these previous data, other notable findings include the observation that whilst steroid precusors such as progesterone and DOC showed an increase in the plasma, this was not the case for downstream steroids such as DHP and DHDOC, where the increase was only found in brain regions, reflecting the observation that neuroactive steroids can be synthesised *de novo* in the brain. This was also the first time that neuroactive steroids such as DHP and DHDOC was quantified after acute stress, and the results showed that these changes following stress were also not uniform across the brain regions, and sex differences were observed. As the biological significance of these results are not a highlight with respect to the aims of this chapter, which focuses on the LC-MS method, these are elaborated in Appendix B.

A

Male Cortex	Sze	Caruso	Vallée	Tobi-ansky	Park	Meffre
Pregnenolone	**1.3**	4.5	0.3	-	1.5	4.9
Progesterone	**1.7**	1.4	-	-	n.d.	0.4
DHP	**0.5**	8.4	-	-	n.d.	0.8
Allopregnanolone	**n.d.**	3.6	0.1	-	n.d.	0.4
Testosterone	**2.2**	4.0	-	0.9	-	1.4
Corticosterone	**10**	-	-	25	10	50.1
DOC	**1.2**	-	-	-	0.1	-

B

Male Plasma	Sze	Caruso	Vallée	Tobi-ansky	Park	Meffre
Pregnenolone	**5.5**	0.4	n.d.	-	n.d.	0.58
Progesterone	**0.7**	0.61	-	-	1.5	2.17
DHP	**2.5**	2.15	-	-	n.d.	n.d.
Allopregnanolone	**0.2**	0.23	0.1	-	0.1	n.d.
Testosterone	**1.0**	3.08	-	1.5	-	1.24
Corticosterone	**32**	-	-	100	120	54.4
DOC	**0.21**	-	-	-	2.5	-

C

Female	Cortex			Plasma		
	Sze	Caruso	Meffre	**Sze**	Caruso	Meffre
Pregnenolone	**2.7**	6.6	9.0	**9.9**	0.8	0.5
Progesterone	**7.2**	11.4	25.0	**9.8**	12.3	25.0
DHP	**1.2**	9.0	50.0	**2.3**	2.2	6.0
Allopregnanolone	**0.6**	4.2	3.0	**3.8**	1.6	3.5
Testosterone	**n.d.**	0.0	-	**n.d.**	n.d.	-
Corticosterone	**19**	-	-	**154**	-	100

D

Male with and without swim stress		Sze		Vallée	
		Basal	**Stress**	Basal	Stress
Pregnenolone	Plasma	**5.5**	**7.0**	n.d	0.1
	PFC	**1.30**	**8.6**	0.3	7.0
Allopregnanolone	Plasma	**0.18**	**0.3**	0.1	1.5
	PFC	**<0.1**	**0.3**	0.1	1.3

Table 3.9: Comparison of neuroactive steroid measurements from Sze et al. (2018) and other studies. Values are in ng/ml for brain measurements and ng/ml for plasma measurements. Concentrations of neuroactive steroids are different between cortex and plasma, and a sex difference can be observed, even at basal conditions (comparing panel A, B with C). Stress also increases the concentrations of neuroactive steroids in both plasma and prefrontal cortex (PFC) (Panel D). Previous studies utilised GC-MS (Meffre et al., 2007, Vallee et al., 2000) or LC-MS (Caruso et al., 2010, Tobiansky et al., 2018, Park et al., 2017) to quantify steroids.

3.4 DISCUSSION

3.4.1 Main findings of this chapter

The aim of this chapter was to develop and validate a method which allows for the reliable and simultaneous quantification of a panel of neuroactive steroids in the rat brain. The LC-MS assay presented in this chapter could successfully quantify 10 out of the 14 steroids initially intended for measurement, namely corticosterone, 11-DHC, DOC, DHDOC, THDOC, progesterone, DHP, allopregnanolone, pregnenolone and testosterone. DHT, 3α- and 3β-diol and oestradiol unfortunately could not be detected using this method, as carbonyl groups (which the GABA modulatory steroids allopregnanolone and THDOC, as well as its precusors possessed) were targeted for derivatisation. Chromatographic peaks were well-separated with good peak shapes, and the accuracy and precision of the assay is within acceptable limits. The method was also biologically validated using rat plasma and brain samples (section 3.3.5; Sze et al., 2018; Appendix B), where it was confirmed that the method can detect expected differences in neuroactive steroid concentrations between different groups (i.e. with and without stress, and in different sexes).

This LC-MS method is valuable in that it allows for the direct examination of changes in concentrations of neuroactive steroids in the rat brain, which is the location in which they exert their actions. Measuring a panel of steroids can also allow for one to investigate how concentrations of different metabolites change in relation to each other. Methods that can quantify a similar, but not identical, panel of neurosteroids have been developed previously, using GC-MS (Meffre et al., 2007) and LC-MS (Park et al., 2017) albeit using different sample processing and detection methods. Values obtained in the biological validation study closely aligned to these previous studies. Whilst most of these previous methods have focussed on quantifying the $GABA_A$-modulating steroids allopregnanolone and THDOC, the method presented in this thesis also additionally considered DHP and DHDOC, the 5α-intermediate of progesterone and DOC, which can allow one to indirectly determine the activity of the enzymes 5α-reductase and 3α-HSD, thereby giving a more complete picture of steroid metabolism in different physiological states.

Most importantly, the successful development and validation of this in-house LC-MS assay within the Roslin Metabolomics Facility is the first step towards elucidating the role of steroids in mediating the outcomes of prenatal stress, the focus of this thesis.

Notably, the biological validation study with acute swimming stress as a manipulation also suggest that it is promising form of acute stress model to use to assess the response of several neuroactive steroids, which laid the foundation of designing studies that incorporate both acute and prenatal stress in the subsequent chapters. Nonetheless, as of all quantification methods, this LC-MS method is not without its limitations, which will be elaborated in this section.

3.4.2 Assay performance

The sensitivity of the assay was adequate to detect almost all neuroactive steroids in the rat brain. Peak shapes were also generally satisfactory at the LOQ, although certain steroids performed better than the others (e.g. LOQ of THDOC was its potential LOD, while corticosterone could have lower LOQs). Any further improvement of the LOQ (sensitivity) may require drastic changes to the instrumentation. For instance, the same C18 column of a smaller internal diameter (0.5 mm) was also tested as part of the method optimisation process. Although the signal intensity was amplified, the change also introduced variability that needed to be compensated by introducing more modifications to the entire instrumental set-up, which was not logistically feasible. For the same reason, other columns such as phenyl columns, which were thought to be able to provide better resolution were also purchased, but were not tested. Ultimately, the main limiting factor in the study is the mass spectrometer that is available for LC-MS analyses in the facility. Whilst the ion trap Amazon ETD mass spectrometer is a robust workhorse instrument, it has shortcomings as compared to more sensitive equipment such as a triple quadrupole instrument, which is able to monitor transitions for a longer period of time, and is more commonly used as the mass spectrometer of choice for such MS/MS targeted analysis.

Three forms of variability were assessed in the validation procedure (section 3.3.4). In general, variability was within limits (15%) for most analytes. As a multistep procedure, it is often difficult to pinpoint the exact points in which variability has been introduced. Injection-to-injection variability suggests that inherent fluctuations in the performance of the LC-MS itself exist, and this can often be due to subtle changes like temperature or ion source pressure, which are difficult or impossible to control (Stokvis et al., 2005). Intra- and inter-assay variation on the other hand suggests that despite precautions, certain aspects of sample processing and derivatisation

may also have contributed to some of the variability. Whilst the intra- and inter-assay variation did not have a cumulative effect on each to other, in general, the inter-assay variation seemed to be greater than intra-variation for most analytes, which accounted for most of the total variation reported for each analyte (Table 3.8).

One of the key problems could have been the stability of the analyte. Steroids are generally heat-stable and can withstand heating of up to 60^{o}C (Taves et al., 2011), and none of the procedures in this LC-MS method involved high heat, therefore it is unlikely that steroids would have been degraded during sample processing. However, the problem could lie with the stability of the final derivatised product. Whilst previous studies have determined that the derivative is stable for 30 min at room temperature (Cobice et al., 2013), batch runs in the LC-MS assays here could take up to 18 hours, therefore it is not known whether the Girard's T derivatives would have degraded in the autosampler on a longer time scale. Nonetheless, in a batch run, effects to minimise this source of variability were taken, where samples were reconstituted and loaded into the autosampler in batches, with different treatment groups well-represented in each batch. Additionally, the purchase of a refrigerated autosampler halfway through the method development process aided sample stability.

3.4.3 Girard's T derivatisation

Girard's T derivatisation was fundamental to the success of the assay. Girard's T reagent is cheap and extremely stable at room temperature, and the reaction was quick and easy to perform, requiring only a 30 min incubation on a heat block. Neutral steroids were converted to positively-charged hydrazones, which augmented their ionisation drastically, giving an intense precursor ion signal. Additionally, when Girard's T hydrazones are fragmented, the specific transition of $[M]^+ \rightarrow [M\text{-}59]^+$ always occurred (Wood, 2017). These advantages have rendered Girard's T derivatisation to be a fairly popular method, and several other studies have also utilised this form of derivatisation (Cobice et al., 2013, Cobice et al., 2016, Dury et al., 2016, Lavrynenko et al., 2013, Johnson, 2005). However, as of all derivatisation agents, the use of it is not without its drawbacks.

Firstly, as can be seen in our chromatogram (Fig 3.9), the Girard's derivatisation reaction is not stereospecific, and *syn*- and *anti*-isomers are introduced, resulting in two peaks. This is not surprising and has been observed in other studies utilising

Girard's reagents (DeBarber et al., 2011, Karu et al., 2007). However, the ratio between the smaller and the larger peak is always the same, and the larger peak is always used for quantification. In our case, the smaller peak did not interfere with any other peaks. The calibration curves were always linear, which indicated that derivatisation reaction occurred in the same manner, with the same stoichiometric ratio, even with differing steroid concentrations.

Secondly, the Girard's T reaction itself although straightforward, poses an inherent problem that could have contributed to the variability of the assay. Whilst the forward reaction of forming a hydrazone required an acid catalyst, the reverse reaction of hydrolysis occurs when a strong acid is present (Wheeler and Rosado-Lojo, 1962). The acid content of the reaction thus needs to be carefully controlled. Optimal catalytic conditions were determined to be 0.2% formic acid, which was strong enough to catalyse the forward reaction, but at the same time, does not allow for too much hydrolysis. Additionally, a neutralising step was added where ammonium hydroxide was added at the end of the 30 min incubation. This not only quenches the reaction, ensuring that yield is consistent in all samples, but also limits the hydrolysis of the formed derivative. The pH was checked to ensure that the final product was neutral.

The third potential problem with Girard's T derivatisation is the possibility of having multiply-derivatised compounds. Some analytes possess three carbonyl groups (11-DHC), and some possess two carbonyl groups (e.g. corticosterone, DOC, DHDOC, progesterone and DHP). However, this has been checked and it was confirmed that no major peak could be detected for double- or triple-derivatised compounds on MS1 mode. Wheeler and Rosado-Lojo (1962) also showed that there were differential reaction efficiencies for different ketone groups (Wheeler and Rosado-Lojo, 1962). The reaction was complete for 3-ketones and $\Delta^{4,5}$-3-ketones, partially complete for 20-ketones and incomplete for 11-ketones. As such, it is probable that mono-derivatisation occurred in each instance, with one only ketone group being preferentially derivatised as long as the acidity is controlled. Studies have indeed shown that with controlled acidity, mono-derivatisation instead of di-derivatisation is likely to have occurred (Johnson, 2005).

While the additional derivatisation steps complicate sample preparation and introduces more potential areas of errors and may not be ideal for detecting all

analytes, it is worth highlighting again that the optimised Girard's T derivatisation in this study enhanced MS2 EIC signals for the steroids of interest more than 100-fold. Thus, these drawbacks associated with derivatisation seem like an inevitable compromise in light of the drastically improved sensitivity.

3.4.4 Specificity

Specificity in detecting an analyte is determined based on three criteria, the retention time, the precursor mass, and the transition. However, steroid isomers exist in the study (e.g. pregnenolone and DHP have the same precursor mass and transition), thus the assessment of specificity for these analytes relies largely on retention time. The retention time was checked for each individual analyte in every sample to ensure that it conformed closely to that of the standard peaks. Each chromatographic peak was also visually inspected to ensure that there are no split or riding peaks, which can indicate the presence of stereoisomers (Ahonen et al., 2013) . Although stereoisomers were not formally investigated in this study, it is likely that Girard's T derivatisation would result in stereoisomers possessing a different elution time from one another. For example, in Dury's method, which used a similar gradient profile to separate Girard's T derivatised steroids, all isomers of pregnanolone (including allopregnanolone, epipregnanolone and epiallopregnanolone) were well-separated without any interference or riding peaks (Dury et al., 2016). Here, in the preliminary experiments using the derivatisation agent DMABC (data not shown), 3α- and 3β-diol could also be successfully separated, adding weight to the concept that LC, with derivatisation, has the ability to separate stereoisomers.

Nonetheless, the detection of a few of these stereoisomers could be a future line of work, as studies have shown that concentrations of stereoisomers might be altered differentially following experimental manipulations. For example, differential concentrations of 3α,5α- and 3α,5β-reduced neuroactive steroids were observed after exogenous administration of neuroactive steroid precursors such as pregnenolone (to rats) and progesterone (to women) (Porcu et al., 2009). It is therefore likely that following an acute stressor, such differences would be observed as well, which could have physiological consequences.

3.4.5 Detection of other remaining steroids

DHT, oestradiol, and 3α- and 3β-diol could not be detected using this Girard's T method. DHT has a carbonyl group, thus it is likely that the prevailing derivatisation and LC-MS conditions were not ideal for its reaction and detection. Formation of a DHT-Girard hydrazone may require a stronger acid catalyst (e.g. 1% TFA) (Tamae et al., 2013), however, 3-ketones are also reported to undergo hydrolysis to a greater rate than the $\Delta^{4,5}$-3-ketones, 17-ketones and 20-ketones (Wheeler and Rosado-Lojo, 1962). Thus, the problem may have arisen from a combination of lower yield from the reaction, but also increased hydrolysis, rendering DHT-Girard T hydrazones undetectable. Thirdly, it may also be plausible that the ionisation method employed in this LC-MS method may not be suitable for the DHT-Girard compound, as the Girard's T derivatised DHT could be detected using mass spectral imaging on tissue sections (Cobice et al., 2016).

Nevertheless, using the workflow in section 3.1.3, the expertise gained from the development of this method can be used in the future to screen and develop a method using other derivatisation agents to detect these other steroids of interest. Dansyl chloride for example, has been widely used to detect oestradiol and its metabolites (Faqehi et al., 2016). In the derivatisation screening experiment, DnCl-derivatised oestradiol indeed show remarkably intense MS1 EIC signals as compared to non-derivatised oestradiol (Table 3.3). However, this was not pursued as the detection of oestradiol alone was not a main focus of this study.

3.4.6 Matrix effects

Matrix effects refer to how the co-eluting components in the biological matrix may alter the behaviour of the analytes of interest, thereby impacting the accuracy of the assay. There are two ways in which matrix effects can be investigated, firstly by comparing the AUC of an analyte in the matrix (e.g. brain tissue) versus the AUC of the analyte in a neat solution (e.g. in methanol only), or by post-column infusion, where the analyte of interest is infused in a steady stream whilst the matrix is being eluted through the column. Points on the elution profile where unexpected dips (i.e. ion suppression) or peaks (i.e. ion enhancement) occur would indicate matrix effects. Although these matrix effects were not formally investigated in this study, efforts were made to reduce the impact of these potential effects in this study by the addition of internal standards.

The ideal internal standard is a stable isotopically labelled analogue of the analyte, usually ^{13}C or deuterium (^{2}H), which elutes at the same time and is subject to the same matrix effects as the analyte (Makin, 2010). Deuterated standards should have three or more deuteriums replacing hydrogens (i.e. D3 and above), in order to prevent interference with naturally-occuring isotopes (Stokvis et al., 2005). Although each analyte should theoretically be accompanied by an isotopically labelled internal standard, this is not always possible in reality. Deuterated internal standards are costly, and may not always be commercially available for every single compound. Moreover, addition of more internal standards increases the possibility of signal interference. For example, DHP-d8 was initially also included as an internal standard in this method, however, as DHP-d8 eluted closely to and had a molecular weight that is only 1 Da away from progesterone-d9, riding peaks were observed where their naturally occurring isotopes interfered with each other. Addition of an internal standard in itself therefore does not guarantee a reliable method and may in fact introduce more inaccuracies and variability.

Therefore, the general consensus is that the use of few internal standards that elute at different parts of the run, with properties corresponding to the properties of a few key analytes, is generally sufficient for a LC-MS method. Upon reviewing the initial method which was published in Sze et al. (2018), two additional internal standards were added to the method, corticosterone-d4, which elutes earlier on in the gradient profile and allopregnanolone-d5, later on in the gradient profile, which should render it more robust than the previously published method.

3.4.7 Sample processing

The sample clean-up method of C18 SPE was better than the Hybrid SPE phospholipid removal method, evident from the greater signal intensity of target EIC peaks, and the decreased presence of contaminants and background noise. One drawback of the C18 SPE method is that the sample processing method is fairly laborious as compared to the Hybrid SPE method, and multiple steps could increase the chance of steroids being lost at each stage. However, the assay validation showed that the recovery was satisfactory (71-108%).

Comparing the starting material required, this method also required far less starting material than several other published methods. The method presented here only required 100 µL of plasma as compared to the 300 µL previously needed to profile a

similar panel of steroids (Porcu et al., 2009). For the rat brain, the minimum starting material required here (20 mg) was also lower compared to some other protocols, some of which required 100 mg of tissue (Caruso et al., 2008). However, recently, a method requiring only 3 mg of starting material was developed in which micro-dissected brain samples of mesocorticolimbic areas was used for LC-MS quantification of steroids (Tobiansky et al., 2018). Although such low amounts of starting material were not attempted here, micro-dissection, which ostensibly offers more regional specificity than the gross dissection used in this protocol, could be a further refinement to the current sample processing and tissue collection method.

3.4.8 Conclusions and future work

Despite these limitations, neuroactive steroids could still be successfully quantified in the rat brain in a physiologically relevant manner (Sze et al., 2018, Appendix B), and the absolute values for most steroids were close to those reported by other laboratories (Table 3.9). Intra-laboratory variations still exist, as LC-MS is far from a perfect technique and its successful implementation requires great care and attention to detail, which may be difficult to standardise across different laboratories (Taylor et al., 2015). Nonetheless, optimisation of the LC-MS method is a reiterative and constantly ongoing process, and various aspects of the method can always be subject to constant modifications and improvements. Although major changes like the changing of mass analysers to newer and more sensitive models are not always financially viable, small changes can be made, e.g. procuring guard columns for the LC-MS to improve column performance.

Given that there is a limit to the number of analytes a targeted analysis approach can reliability quantify, a possible next step could be to extend this targeted steroid profiling to an untargeted omics strategy to study steroid metabolism in a more holistic manner. Although a MS-based “steroidomics” requires a different workflow and also the processing of large datasets, certain aspects such as sample processing to extract steroids, LC separation and MS detection are all based on the same fundamental understanding of steroid chemistry and the basic principles of LC and MS that this work was based on (Jeanneret et al., 2016).

As a concluding note, even with precautions in place to ensure better performance of the method, a much more complex issue at the heart of LC-MS quantification in biological applications is that experimental manipulations or physiological states

would not only alter the analyte of interest, but may also alter the concentration of any co-eluting compounds. The nature of LC-MS means that even with the best equipment and ability to quantify multiple analytes, there is always an inevitable and disconcerting possibility that differences could be caused by other unknown compounds augmenting the signal intensity of the analytes of interest, rather than changes in the actual concentrations of the analytes of interest *per se*. Therefore, rather than attempting to develop an impeccable method, it is perhaps more practical to place these measurements in a biological context and focus on interpreting the results with these caveats in mind, instead of taking them at face value. The rest of the thesis is therefore focussed on the application of this method to quantify steroids and thereby investigate their role in the regulation of different physiological states (e.g. following acute stress or the long-term effects of prenatal stress)

Chapter 4: Effects of prenatal stress on neuroactive steroids concentration following acute swim stress

4.1 INTRODUCTION

In the previous chapter, a LC-MS method was developed and validated for the quantification of a panel of neuroactive steroids. In this chapter, the method was used to quantify neuroactive steroids in the plasma and brain of control and PNS offspring, with and without an additional acute stressor. The acute stressor used in this chapter was swimming stress, replicating the procedure used in the biological validation study in the previous chapter.

4.1.1 Conceptualisation of this study

The conceptualisation of this study was based on three previous observations from experiments in the Brunton laboratory. These observations, summarised in Figure 4.1, were: (i) HPA axis regulation is altered in PNS offspring, (ii) Neuroactive steroid concentrations increase rapidly and robustly following acute swim stress, and (iii) neuroactive steroid production seems compromised in PNS offspring, and treatment with neuroactive steroids rescues the HPA axis dysregulation present in PNS offspring. This led to the hypothesis that HPA axis dysregulation in the PNS offspring could be explained by compromised levels of neuroactive steroids in the brain at baseline, and following acute stress.

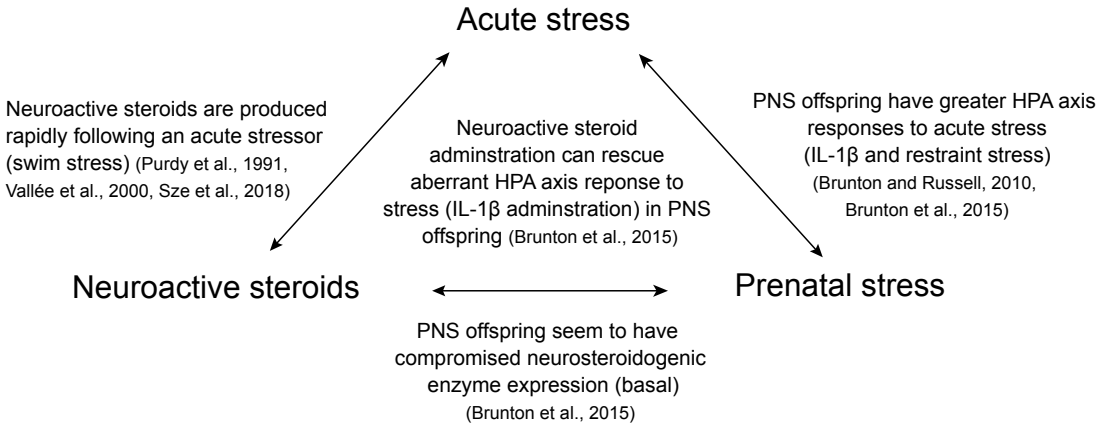


Figure 4.1: Relationship between prenatal stress, responses to acute stress and neuroactive steroids. While the relationship between these three factors have been determined in separate studies, neuroactive steroids have never been directly quantified in the PNS offspring, either basally or following an acute stressor, and not in the same experiment.

4.1.1.1 HPA axis regulation is altered in prenatally stressed (PNS) offspring:

HPA axis dysregulation has been well studied in this prenatal social stress model, mainly through repeated blood sampling experiments where one could obtain time-course information on plasma ACTH and corticosterone concentrations following an acute stressor. In general, plasma ACTH and corticosterone response to acute stressors in adulthood are greater in the PNS offspring, in response to systemic IL-1β administration, a physical stressor (Brunton et al., 2015, Brunton and Russell, 2010), and restraint stress, a psychogenic stressor (Brunton and Russell, 2010).

In these previous studies, there were no differences in basal ACTH and corticosterone secretion between male control and PNS rats. In females, the pattern was less clear, as female PNS rats had higher basal ACTH and corticosterone concentrations in some experiments (Brunton and Russell, 2010), but not in others (Brunton et al., 2015). Following both forms of acute stress, the absolute concentrations of ACTH and corticosterone were always greater in PNS groups as compared to control groups, in both males and females. As repeated blood sampling was carried out, the amplitude of the response (i.e. delta value from baseline) and the duration of the response could also be investigated. Both the amplitude and the duration of the ACTH and corticosterone response were greater in PNS males compared to controls. In females, although the absolute values of ACTH and corticosterone concentrations were always higher in PNS groups, there were instances where the amplitude (i.e. delta value) of the corticosterone response was not always greater than controls, possibly due to the higher baseline levels (Brunton et al., 2015).

Discrepancies between studies are likely due to individual differences in stress responses in the dam following social stress, which then translates to possible variations in the epigenetic programming of the offspring prenatally, as well as possibility of variations in post-natal maternal care, leading to differential outcomes in the offspring during adulthood (Claessens et al., 2011, Boersma and Tamashiro, 2015). Although these previous experiments were carried out in the same facility, they were carried out in different time periods with different batches of rats. It is possible that between these batches of rats, subtle environmental differences were present, which can result in different microbiota in different groups of rats, which is a strong influencing factor that may may inadvertently affect corticosterone

concentrations both basally and in response to stressors (Franklin and Ericsson, 2017).

4.1.1.2 Neuroactive steroid concentrations are rapidly increased following acute swim stress in rats

Forced swim was shown in the previously published study (Sze et al., 2018) as well as in several other rat studies (Vallee et al., 2000, Purdy et al., 1991) to induce a robust increase in both corticosterone and other neuroactive steroids in the brain of control rats. Acute swim stress is a potent combined physical and psychogenic stressor that results in various physiological, endocrine, and immune changes (Abel, 1993, Connor et al., 1997). Previous studies have reported an increase in plasma corticosterone and allopregnanolone within 5 min of the onset of a swim stress session, along with an increase in plasmatic prolactin, ACTH, and glucose (Abel, 1993, Purdy et al., 1991, Armario et al., 1995). Swim stress also results in widespread activation of various brain regions, including stress responsive nuclei in both the forebrain and the hindbrain (Cullinan et al., 1995).

In the previous acute swim stress experiments, the increase in allopregnanolone and pregnenolone in the brain also occurred prior to the increase in the plasma, indicating that these neuroactive steroids are likely to be produced within the brain after stress rather than entering from the periphery (Purdy et al., 1991). It appears that whilst upstream neuroactive steroids such as progesterone, DOC, and corticosterone are produced by the adrenal gland and then transported into the brain, $GABA_A$-active steroid metabolites are produced *de novo* in the brain, as strong correlations between the plasma and the brain were observed for the precursors but not the metabolites (Sze et al., 2018). Although it is not known whether acute swim stress would result in similar hyperactivity of the HPA axis in PNS offspring, for these above reasons, forced swim was used to induce stress in this study.

4.1.1.3 Neuroactive steroid production may be compromised in PNS offspring

Alterations in neuroactive steroid production could be one of the mechanisms by which the aberrant HPA axis responses to stress in the PNS offspring occur. In previous studies carried out using the prenatal social stress model (Brunton et al.,

2015), three lines of evidences suggested that neuroactive steroid production could be affected in the brains of males and female PNS rats.

Firstly, mRNA expression of 5α-reductase type 1 was altered. In males, PNS offspring had decreased 5α-reductase type 1 mRNA expression in the PVN and the NTS, but increased expression in the prefrontal cortex, the islands of Calleja (ICj) in the ventral striatum, and dorsal part of lateral septum as compared to control males (Brunton et al., 2015). In females, a similar decrease in 5α-reductase type 1 mRNA expression was observed in the NTS, whilst an increase in the ICj was observed (Brunton et al., 2015). 3α-HSD mRNA expression on the other hand, was not affected in either males or females in any of the brain areas investigated.

Secondly, upregulating 5α-reductase type 1 and 3α-HSD mRNA expression in the NTS via adenoviral-mediated injection to PNS female rats rescued the aberrant HPA axis ACTH and corticosterone responses to IL-1β administration (Brunton et al., 2015). Thirdly, aberrant HPA axis responses to IL-1β administration were also rescued when neuroactive steroids were administered 20 and 2 hr prior to acute stress exposure (female rat offspring were pre-treated with allopregnanolone, whilst male rat offspring were pre-treated with 3β-androstanediol) (Brunton et al., 2015).

Together, these observations indicate that in this model of prenatal stress, there appear to be deficits in neuroactive steroid production (especially the 3α,5α-reduced $GABA_A$ positive modulatory steroids) in the offspring following stress exposure, which may underlie the heightened ACTH and corticosterone responses following IL-1β administration. However, neuroactive steroid concentrations have never been directly measured. It was therefore the aim of this study to measure neuroactive steroid concentrations in the brain of control and PNS rats under basal conditions and following exposure to acute stress, in tandem with plasma corticosterone (as a readout of the HPA axis), to reveal if there are indeed deficits in neurosteroidogenesis that may be related to the changes in steroidogenic enzyme expression mentioned above.

4.1.2 Neuroactive steroids investigated in this chapter

The nine steroids quantified in this study were: corticosterone, DOC, DHDOC, THDOC, progesterone, DHP, allopregnanolone, pregnenolone and testosterone. The significance of quantifying each steroid in the plasma and the brain, with respect to stress responses, are reviewed in this section.

Corticosterone: Plasma corticosterone was quantified in this study as a readout of HPA axis responsiveness. Brain corticosterone concentrations were also quantified as they have a role in various processes such as activation of the HPA axis, negative feedback mechanisms, or memory processing, depending on the brain region (Osborne et al., 2015, Sapolsky et al., 2000). In the rat brain, most of the corticosterone in the brain is of adrenal origin, although local concentrations can also be altered by the action of 11β-HSD1 and 11β-HSD2 (section 1.3.2.2) (Ye et al., 2008). It has been established that female rats generally have higher concentrations of basal plasma corticosterone, and also secrete larger amounts of corticosterone following acute stress (Sze et al., 2018, Handa et al., 1994, Tinnikov, 1999). In control rats, it has been established that despite sex differences in the plasma, it did not seem like sex differences were present in the brain following stress, except for the brainstem (Sze et al., 2018), probably due to females having greater CBG concentrations (Gala and Westphal, 1965).

Pregnenolone: Pregnenolone was quantified in this study as it is the precursor of all neuroactive steroids, and any changes in pregnenolone concentrations may point to changes in the efficacy of the p450scc enzyme, and may have consequences on levels of downstream steroid metabolites. It has been difficult to draw conclusions about pregnenolone's effect on the HPA axis from experiments involving the exogenous administration of pregnenolone or trilostane (a 3β-HSD inhibitor) to rats, as its effects are confounded by both its metabolism to excitatory steroids (such as pregnenolone sulfate and DHEA), and also inhibitory steroids such as $GABA_A$-enhancing progesterone metabolites (Naert et al., 2007). Nonetheless, pregnenolone itself is generally considered to be inactive on $GABA_A$ receptors (Harrison et al., 1987). In previous studies on control rats, acute swim stress did not increase pregnenolone levels in the plasma, however, an robust increase was observed in the brain (Vallee et al., 2000, Sze et al., 2018), and a sex difference

exists where females show greater pregnenolone concentrations in all brain regions (Sze et al., 2018) (Appendix B).

Deoxycorticosterone (DOC): Deoxycorticosterone was quantified in this study as it is able to participate in stress responses possibly through binding to MR and GR, and also by conversion to the downstream $GABA_A$-enhancing metabolites DHDOC and THDOC, which are involved in stress responses (Reddy, 2006). DOC concentrations are increased robustly in both the plasma and in the brain following swim stress in control rats (Sze et al., 2018) (Appendix B). Similar to corticosterone, its secretion is also under the control of ACTH and it is produced mainly in the zona fasciculata of the adrenal gland (Reddy, 2006, Tan and Mulrow, 1975). It is known to have glucocorticoid properties and can bind to the GR (Reddy, 2006), has anti-convulsant properties when administered to rats, and may potentially modulate stress-sensitive conditions in humans such as panic disorder, PTSD, major depression, and epilepsy (Sapolsky et al., 2000, Reddy and Rogawski, 2002, Reddy, 2006). Additionally, it is considered a mineralocorticoid which involved in the regulation of sodium balance and blood pressure (Vinson, 2011).

DHDOC: DHDOC is the 5α-reduced intermediate of DOC, and was considered in this study as alterations to its concentrations could signify altered efficacy of 5α-reductase (Fig 1.3). DHDOC is a known agonist of PR (Rupprecht et al., 1993), and may also act as a positive allosteric modulator of the $GABA_A$ receptor (Reddy and Rogawski, 2002). Similar to its precursor DOC and metabolite THDOC, DHDOC also has anti-convulsant properties in the rat (Reddy and Rogawski, 2002). In control rats, it has been previously been reported that greater levels of DHDOC is only observed in the brain and not in the plasma following acute swim stress (Sze et al., 2018) (Appendix B), indicating that it is likely to be synthesised *de novo* in the brain following an acute stressor.

THDOC: THDOC was investigated in this study as it is a positive allosteric modulator of $GABA_A$ receptors and can alter inhibitory transmission that are involved in the stress responses. The administration of THDOC to rats attenuates stress-induced increases in plasma corticosterone (Owens et al., 1992), whilst electrophysiology experiments in mpPVN neurones have demonstrated a role for THDOC in decreasing sympathetic output (Womack et al., 2006). Basally, THDOC has been found to be involved in tonic inhibition of the HPA axis, however, it has

also been reported to be involved in the activation of the HPA axis in mice, via actions on $GABA_A$ receptor subtype δ on CRH neurones (Sarkar et al., 2011). It is thus suggested that neurosteroids like THDOC (and perhaps allopregnanolone) not only have a role in dampening the HPA axis response, but may also be required to mount a HPA axis response to stress (Sarkar et al., 2011).

Progesterone: Progesterone was investigated in this study as it has complex interactions with the HPA axis and glucocorticoids (Wirth, 2011). Apart from binding to PR and having a generally inhibitory effect on the HPA axis (Section 1.3.4), progesterone is also able to bind to GR, acting as an antagonist to reduce GR-glucocorticoid binding and therefore impairing feedback (Svec, 1988). Furthermore, progesterone is the precursor to downstream metabolites DHP and allopregnanolone, which regulate the HPA axis in their own manner (Wirth, 2011). Progesterone is produced rapidly following stress alongside corticosterone from the adrenal cortex, and this is observed even in male rats and in ovariectomised female rats, suggesting the contribution of the adrenal gland (Hueston and Deak, 2014), possibly also from CRH- and ACTH-mediated mechanisms (Torres et al., 2001). A sex difference is found previously in control rats, where females show greater plasma progesterone concentrations at baseline, and also elevated concentrations following stress compared to males, in both the plasma and various brain regions, especially the hypothalamus (Sze et al., 2018) (Appendix B).

DHP: DHP was investigated as it is the intermediate between progesterone and allopregnanolone, and it also has rapid effects on the brain, exerting its effects possibly through binding to membrane-bound PR, which it has a high affinity for as compared to intracellular PRs (Singh et al., 2013). Baseline levels of DHP in the mouse brain were found to be decreased under prolonged stress (e.g. social isolation), which possibly reflects its ability to modulate changes in gene transcription following long-term stress (Dong et al., 2001). However, it has recently been proposed to also have anti-seizure properties in rats (Wu and Burnham, 2018). Plasma DHP does not increase following acute swim stress in control male and female rats, however, DHP was increased in the brain following acute swim stress, and females again had greater DHP concentrations in most regions of the brain (Sze et al., 2018) (Appendix B).

Allopregnanolone: In contrast to its precursors progesterone and 5α-DHP, allopregnanolone does not bind to PR, but like THDOC, allopregnanolone is a positive modulator of $GABA_A$ receptors, playing an important role in stress responses (Morrow et al., 1987). Allopregnanolone is one of the most well-characterised neuroactive steroid, especially since the discovery that it increases robustly in the brain following acute swim stress in rats, often with a slower or a negligible increase in the plasma, suggesting *de novo* synthesis in the brain (Purdy et al., 1991, Vallee et al., 2000, Sze et al., 2018). Studies which employ exogenous allopregnanolone administration have determined that allopregnanolone attenuates stress-induced HPA axis activity in rats, preventing the increase in plasma ACTH and corticosterone following acute stress (Patchev et al., 1996). When administered to PNS females offspring in the prenatal social stress model, allopregnanolone normalises stress-induced HPA axis hyperactivity (Brunton et al., 2015).

Testosterone: Testosterone is the principal male sex hormone produced by the Leydig cells of the testes, whilst in females, small amounts are also produced by the ovaries and the adrenal cortex (Burger, 2002). Testosterone was investigated in this study due to its involvement in determining sex differences in HPA axis responses. Testosterone administration to rats and mice has been shown to play an inhibitory role over HPA axis responses to stress, either via a medial preoptic area (mPOA)-mediated pathway or through a serotonin signalling pathway (Viau and Meaney, 1996, Goel et al., 2011). Although these effects may be mediated by rapid non-genomic testosterone actions on AR (Deng et al., 2017), many of testosterone's rapid effects also arise through the action of its metabolites (e.g. DHT, 3α- and 3β-diol) and their effects on other receptors (Foradori et al., 2008). For instance, 3α-diol is known to mediate anti-anxiety effects through a $GABA_A$-mediated pathway (Edinger and Frye, 2005, Frye et al., 1996), whereas 3β-diol could alter HPA axis activity via an oestrogen receptor-β (ERβ) and oxytocin-mediated mechanism (Handa et al., 2009, Lund et al., 2006, Foradori et al., 2008) Unfortunately the metabolites of testosterone could not be quantified using the LC-MS method developed here (for reasons explained in Chapter 3). In the prenatal social stress model, PNS male rats had significantly greater plasma testosterone concentrations in one study (Brunton et al., 2015) but not the other (Ashworth et al., 2016). However, it is not clear whether testosterone concentrations in the brain were altered. Additionally, testosterone concentrations in the plasma and brain were not modified with acute swim stress in control rats (Sze et al., 2018) (Appendix B).

4.1.3 Brain regions investigated in this study

Given the heterogeneity of the brain, regional differences in steroid concentrations exist (Sze et al., 2018), and these differences are known to occur even in early developmental stages in the rat brain (Konkle and McCarthy, 2011). Similarly, regional differences have also been observed in post-mortem human brain tissue (Bixo et al., 1997). These differences can arise from the differential uptake of steroids from the circulation (Karavolas et al., 1976, Bixo and Backstrom, 1990), or differing rates of local metabolism due to varying expression levels of neurosteroidogenic enzymes (Konkle and McCarthy, 2011, Bixo and Backstrom, 1990). The five brain regions investigated in this study have key roles in modulating the stress response (Fig 4.2), and a brief introduction of each region in relation to its role in stress response regulation is given in this section.

In brief, the hippocampus and amygdala and the prefrontal cortex are limbic areas that together process stressful experiences, and subsequently interface with other more primitive brain areas such as the hypothalamus and brainstem to produce a response that copes with the stressor (McEwen and Gianaros, 2010). Whilst each brain region are important in modulating stress responses in their own right, the level of involvement of each brain area can be different depending on the type of the stressor, and also other factors such as temporal dynamics. For instance, it has been proposed that changes in the amygdala precedes the hippocampus following a stressor (Tottenham and Sheridan, 2009). Additionally, stress can affect different regions differently, and acute stress can impair PFC function but instead enhance amygdala function (Arnsten, 2009). Moreover, it is worthy to note that the contribution of each structure to the regulation of the stress response is also subject to finer regulation from its sub-regional structures and also the nature of stressor (e.g. psychological or physical) administered. Additionally, although each region is reviewed separately, there exists cross-talk between these brain regions, adding another layer of complexity that can affect the final information input (Ulrich-Lai and Herman, 2009).

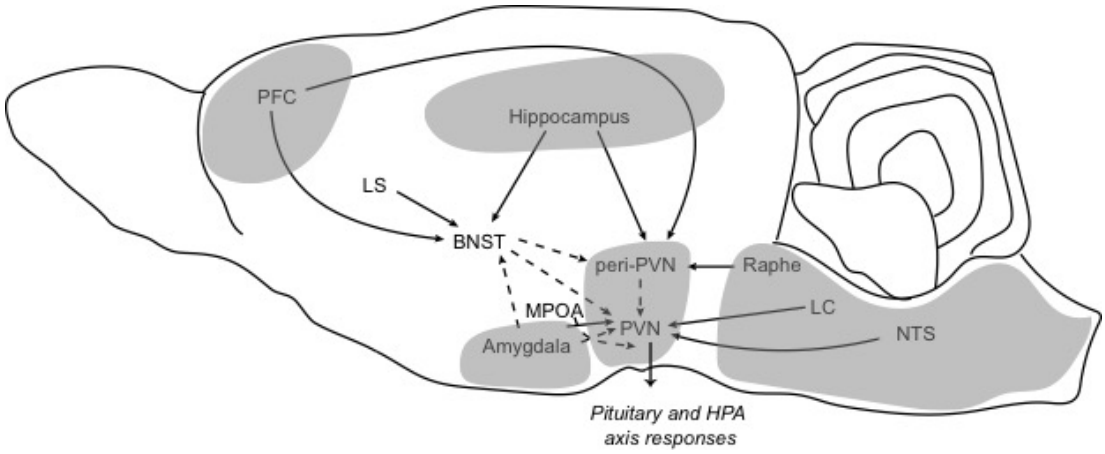


Figure 4.2: Overview of the brain regions investigated in this study. Limbic (i.e. hippocampus, amygdala and frontal cortex) and brainstem structures project to the PVN to enhance or inhibit HPA axis activity. Solid lines represent excitatory projections whilst dashed lines represent inhibitory projections. In general, systemic stressors that are a direct threat to the survival of the organism have been associated with direct signalling pathways from the midbrain or brainstem to the PVN, whilst psychogenic stressors rely more heavily on the limbic system (Jankord and Herman, 2008, Herman et al., 2005). Abbreviations: PVN: paraventricular nucleus, PFC: prefrontal cortex, LS: lateral septum, BNST: bed nucleus of the stria terminalis, mPOA: medial preoptic area, LC: locus coeruleus, NTS: nucleus of the solitary tract, Raphe: dorsal and medial raphe nuclei. Schematic representation not drawn to the scale of any particular sagittal plane, adapted from (Heck and Handa, 2019).

Frontal Cortex: The frontal cortex was investigated as it is the higher order brain region that regulates the processing of stressful signals (reviewed in (Arnsten, 2009)). The prefrontal cortex has abundant GR-containing neurones, and these neurones are activated following restraint stress in male rats (Ostrander et al., 2003), suggesting that the frontal cortex is a direct target of stressful stimuli, at least in the case of psychogenic stressors. Corticosterone in the frontal cortex is thought to exert a net inhibitory effect over the HPA axis, as corticosterone implants decrease ACTH and corticosterone responses to restraint stress in rats, whilst lesions increase HPA axis responses to stress (Diorio et al., 1993). However, even within the frontal cortex itself, different sub-regions can contribute to different aspects of stress output. In the rat, the prelimbic prefrontal cortex (PFC) has been reported to inhibit autonomic stress responses, especially in terms of response termination, whilst the infralimbic PFC on the other hand, is generally associated

with the initiation of autonomic and HPA axis responses (Ulrich-Lai and Herman, 2009, Figueiredo et al., 2003b). Additionally, there seems to be evidence of stressor-specific regulation, where the prelimbic PFC seems to inhibit responses to restraint stress but not systemic stressors, whilst the infralimbic PFC has the ability to limit HPA axis responses to systemic stressors, but initiate HPA axis responses to psychogenic stressors like restraint (Radley et al., 2006, Figueiredo et al., 2003b, Crane et al., 2003, Herman et al., 2005). As an added layer of complexity, the frontal cortex also has various different interconnections with the hippocampus and amygdala, which can further modify the final stress output (McKlveen et al., 2015).

Amygdala: The amygdala is involved in fear processing, mediating social behaviour and anxiety, plays a critical role integrating autonomic and neuroendocrine stress responses. Activation of the amygdala is generally associated with HPA axis activation (Smith and Vale, 2006). However, like the PFC, there exists substantial sub-regional differences, and the final output is also stressor-specific (Dayas et al., 1999). The CeA for example, densely innervates NTS and PVN-projecting structures such as the BNST (Herman et al., 2005) and is especially sensitive to systemic stressors such immune challenge (Xu et al., 1999). The CeA contains large numbers of CRH neurones, and activates the HPA axis through CRH-mediated pathways (Gallagher et al., 2008). Conversely, increased corticosterone in the brain can increase CRH in the amygdala, which is linked to an anxious phenotype in rat studies (Schulkin et al., 1998).The MeA, which has some direct projections to the PVN but also heavily innervates the BNST, is activated following stimuli such as noise, restraint, and forced swim stress (Dayas et al., 2001) but not by systemic stressors (Dayas et al., 1999). Various sub-regions of the amygdala (e.g. CeA and BLA) are also comprised of GABA neurones, implying that GABAergic signalling plays an important role in the amygdalar regulation of stress responses (reviewed in (Jie et al., 2018)).

Hippocampus: The hippocampus show abundant expression of both GR and MR (Reul and de Kloet, 1986) and is involved in the negative feedback inhibition of the production of glucocorticoids (Jacobson and Sapolsky, 1991), therefore exerts a predominantly inhibitory influence over the HPA axis. Additionally, its inhibition is also stressor-specific, for instance, lesions of the hippocampus enhance corticosterone responses to psychogenic stressors such as restraint and novelty, but the response to systemic stressors like ether inhalation seems unaffected

(reviewed in (Jankord and Herman, 2008)). Therefore, like the frontal cortex and amygdala, the stress response following activation of the hippocampus is stressor-dependent, but the final output is largely inhibitory.

Hypothalamus: The role of the hypothalamus in the HPA axis has been introduced in depth Chapter 1, where it is the centre of stress signal integration in the brain (Herman and Tasker, 2016). The hypothalamus itself consists of various sub-regions and these connections between sub-regions (e.g. from the peri-PVN or dorsomedial hypothalamus to the PVN) are mostly controlled by local GABAergic networks (Cullinan et al., 2008). The sexually dimorphic nucleus of the medial preoptic area (mPOA) is also located in the hypothalamus and expresses high levels of AR. Whilst it is known to be of a larger in volume in males of many species, including humans and rodents, and its volume can be impacted in event of prenatal stress (Kerchner and Ward, 1992, Swaab and Fliers, 1985).

Brainstem: The brainstem plays an integral role in autonomic signalling following stress, and its activation generally results in HPA axis activation. Apart from direct NTS signalling to the PVN through noradrenergic and adrenergic signals (Plotsky et al., 1989), the locus coeruleus, also located in the brainstem, is also involved in arousal and also projects to the amygdala, PFC and hippocampus, thereby adding an additional layer of complexity in the brainstem regulation of stress (Ziegler and Herman, 2002). The role of corticosterone in the brainstem has largely been shown to be excitatory (Tasker et al., 2005), it could be affected by sub-regional differences and the nature of the stressor. For instance, the effect of corticosterone is primarily excitatory in the locus coeruleus, but in the reticular formation, the effect of corticosterone on caudal and rostral reticular formation neurones seems to be conflicting (Avanzino et al., 1983). In another example, lesions to the ventral noradrenergic neurones inhibits corticosterone responses to ether inhalation but not to restraint stress (Ziegler and Herman, 2002), whilst lesions to medullary projections blocks neuronal activation following IL-1β injection but not footshock (Li et al., 1996). The brainstem is deemed to be of particular relevance to the prenatal social stress model, as decreased 5α-reductase type 1 mRNA expression are particularly obvious in the NTS of PNS rats from both sexes (Brunton et al., 2015).

4.1.4 Aims of this chapter

Using the method developed in Chapter 3, this study aims to obtain a steroid profile for the plasma and the brains of control and prenatally stressed male and female offspring, under acute stress and non-stress conditions. The panel of steroid and brain regions have been chosen due to their involvement in the stress response, as explained in section 4.1.2 and 4.1.3, respectively. Swimming stress was used as the acute stressor as it has previously been shown to result in a robust increase in neuroactive steroids, explained in section 4.1.1.2 and in Sze et al., 2018. The specific aims of this study were to determine:

1) Whether HPA axis hyperactivity is observed following acute swim stress in PNS offspring, by comparing plasma corticosterone concentrations between controls and PNS
2) Whether there are any differences in neuroactive steroid concentrations between controls and PNS rats under basal conditions and following acute stress, in the plasma and the various brain regions; and
3) Whether there are sex differences in the neuroactive steroid response, especially in the PNS rats

The hypotheses of this chapter are:

1) Following acute swim stress, plasma corticosterone concentrations are greater in PNS offspring as compared to control offspring
2) Concentrations of neuroactive steroids, especially $GABA_A$ modulatory neurosteroids and their precursors are lower in the all brain regions of PNS offspring, both basally and following acute stress, leading to a general loss of inhibitory tone, therefore greater and more prolonged HPA axis activation and greater plasma corticosterone concentration in the PNS offspring (as in point 1)
3) Females mount a greater stress response (i.e. greater amount of plasma corticosterone as compared to males) and may produce greater amounts of neuroactive steroids as compared to males following acute stress. Prenatal stress may affect this sex difference between females and males.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Control and prenatally stressed (PNS) offspring were generated in the Roslin Institute Biological Resource Facility. Twenty experimental female rats were purchased from Charles River (Margate, Kent, UK), and ten of them underwent social stress from gestational days 16 - 20 using the resident intruder paradigm, as described in section 2.3.1.

Following birth, the offspring stayed with their mothers until the day of weaning (PND23), where one rat of each sex from each litter was tail-marked and regrouped into individually ventilated cages of ten rats each. Rats were group-housed with other rats of the same prenatal stress status (i.e. controls or PNS). For the purposes of this experiment, this generated 8 cages of 10 rats each (2 cages each of control males, PNS males, control females, PNS females). Weaned offspring were left undisturbed, except for routine husbandry as well as some light handling once a week, for the marking of tails until the commencement of the experiment.

4.2.2 Acute swimming stress

When animals were 7 weeks old (between 49-52 days old), an acute stressor was given to half of the groups. Swim-stressed rats were placed in a glass cylinder (diameter, 25cm; height, 50cm) filled with water (22-23^{o}C) to a depth of 30 cm. After 2 min of swimming, they were gently dried in a towel and then returned to their home cage. Swim-stressed rats were culled 30 min after the onset of swimming stress via conscious decapitation (Chapter 2) in a separate room, for the collection of trunk blood and brain tissue. Non swim-stressed (or basal) groups were undisturbed and brought directly into the separate room for culls. Swimming stress was carried out with the help of PhD student Daniela Schnitzler, while I performed the conscious decapitation and tissue collection. Swimming stress and the killing of rats was carried out between 10:00 – 14:00h The experiment was carried out within a 1 week period, with 16 rats culled each day (Table 4.1).

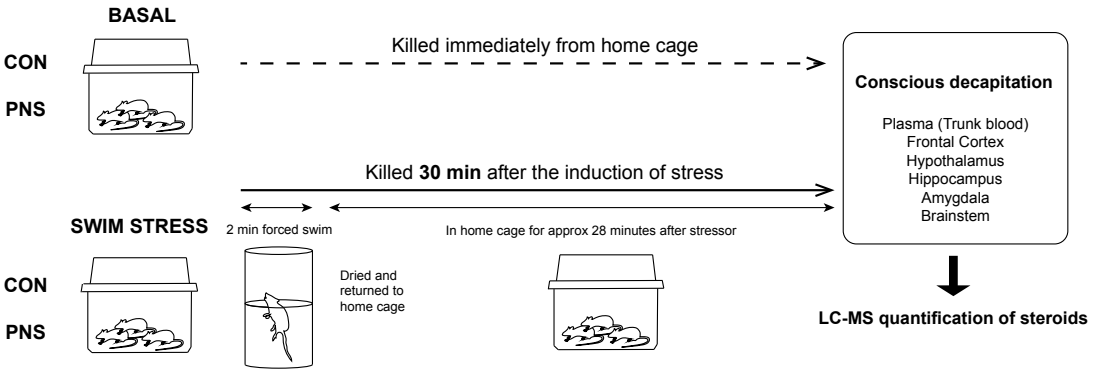


Figure 4.3 Pictorial representation of experimental design. Four groups of rats of each sex was used. Controls and PNS rats, with and without swim stress were housed separately. Rats that did not undergo swim stress were killed immediately from the home cage, while rats that underwent swimming stress were returned to their home cage and killed 30 min after the induction of stress.

Cage	Rats	Day 1	Day 2	Day 3	Day 4	Day 5
1	Male, Control, No Swim/Basal	4	4	2		
2	Male, Control, Swim Stress	4	4	2		
3	Male, PNS, No Swim/Basal	4	4	2		
4	Male, PNS, Swim Stress	4	4	2		
5	Female, Control, No Swim/Basal			2	4	4
6	Female, Control, Swim Stress			2	4	4
7	Female, PNS, No Swim/Basal			2	4	4
8	Female, PNS, Swim Stress			2	4	4

Table 4.1: Number of rats in each group and number of rats killed each day.

4.2.3 Tissue collection and gross dissection

Upon conscious decapitation, trunk blood (ca. 5 - 6 ml) was collected in a tube containing 0.5 ml 5% w/v EDTA and kept on ice until centrifugation at 4^{o}C to separate the plasma. Plasma was stored at -20^{o}C until steroid measurement using LC-MS was performed. The skull was then removed to expose the brain (section 2.3). Whilst still in the skull, the brain was cut in front (Bregma -8.00) and at the back of the cerebellum, separating the hindbrain from the forebrain and midbrain. Both blocks were removed from the skull with care, and were bissected along the midline. In this experiment, the left hemisphere of the brain was frozen on dry ice for future use, while the right hemisphere was subjected to gross dissection (Fig 4.2). The right cerebellum was removed and discarded, leaving the right brainstem, which includes both the pons and the medulla. The cerebral cortex was first peeled open

to expose the hippocampus, which was separated out easily. The cortex was then placed back into position and the frontal cortex excised (at Bregma 2.00 mm). The brain was then flipped to the ventral side, and a block was made by cutting along Bregma -1.60 mm and Bregma -5.60 mm with a scalpel, using the optic chiasma as an anatomical landmark. The hypothalamus and amygdala were then separated, using the rhinal fissure and optic tract as anatomical markers. Dissected brain regions were placed in a 1.5 mL Eppendorf tubes and frozen immediately on dry ice. They were transferred to -80^{o}C for storage until sample processing for LC-MS.

4.2.4 Sample processing and LC-MS

Sample processing was carried out as described in chapter 2 (section 2.5). Weights of brain regions from the right hemisphere were taken before homogenisation, while 100 µL of plasma was used for sample processing. Samples from all four treatment groups, from the same region and sex (n = 40 in total), were processed on the same day, alongside 7 standard calibrants and a blank. Samples from males and females were processed on separate days. Calibration curves were diluted from 250 ng/mL aliquots of a master stock solution containing nine steroids. Due to the laborious process of sample homogenisation and clean-up, the process was staggered and 12 – 18 samples were handled at any one time. The derivatisation reaction was carried out at the same time for all samples within a batch.

For LC-MS analysis, samples from all four stress conditions the same region, and the same sex, were analysed on the same day, using the same standard calibrants and same batch of reagents and solvents. A batch run was set up, where standard calibrants were always first to be injected and analysed. Duplicate injections were carried out for all calibrants and samples. Due to the length of the run and the number of samples, samples were reconstituted and loaded into the autosampler in sample subsets, and each subset had equally divided groups. Data acquisition and analysis for LC-MS was carried out as described in chapter 2.

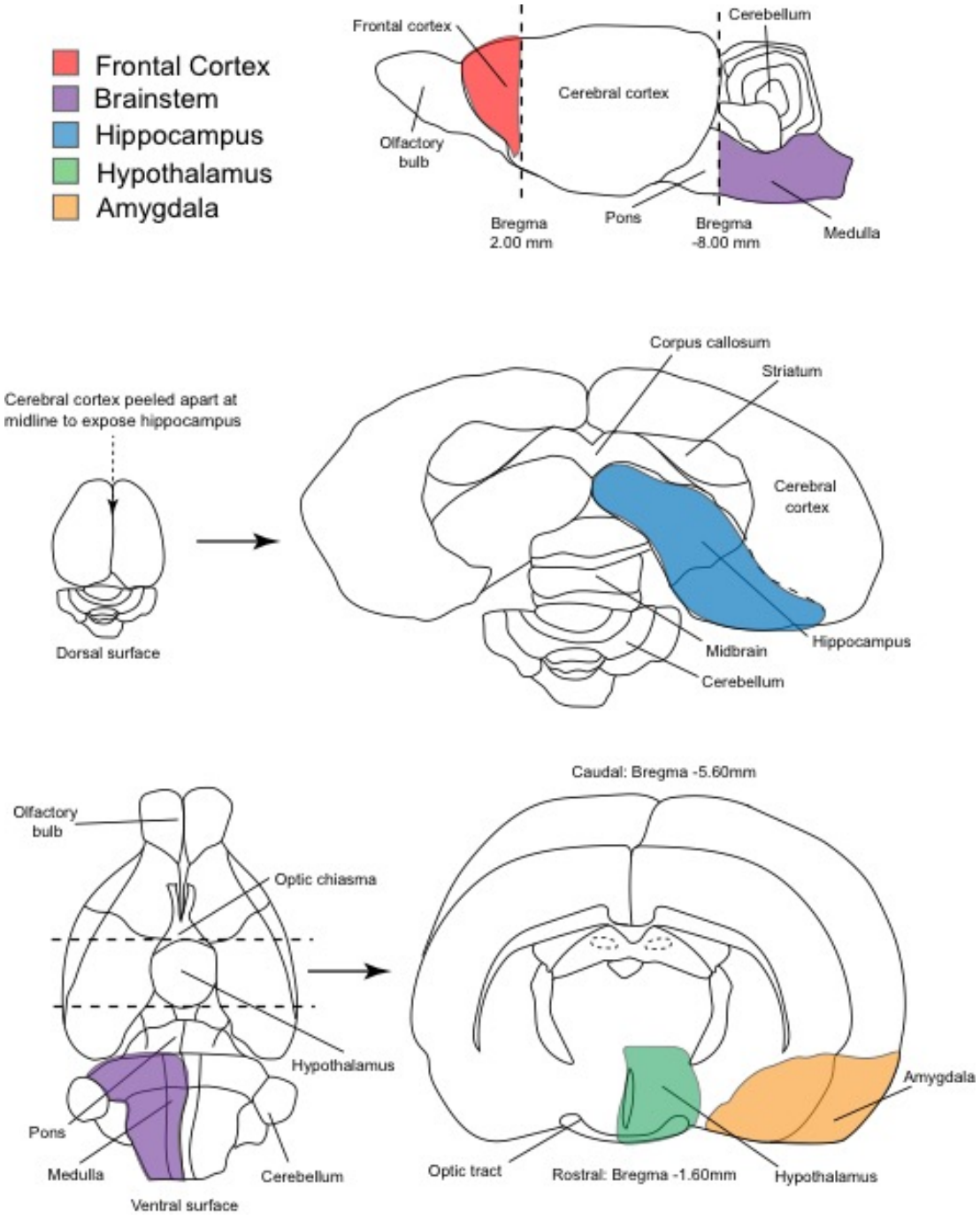


Figure 4.4: Brain regions collected for LC-MS quantification of steroids. Gross dissection was carried out on one hemisphere of the brain, where regions were collected according to the anatomical landmarks outlined in the figure. (Adapted from supplementary figure 1, Sze et al., 2018)

4.2.5 Data Analysis

Although there were three factors involved in this study (acute stress, prenatal stress and sex), the main aim of the experiment was to study the effects of prenatal stress on neuroactive steroid concentrations, with and without an additional stressor during adulthood. As it is known that steroid concentrations are different in males and females, it was planned for the two sexes to be analysed separately by the use of two 2X2 ANOVAs, where prenatal stress and acute stress were the two main factors. Significance level was set at 0.05. Post-hoc testing utilising Student-Newman-Keuls multiple pairwise comparisons were also carried out, using R-studio (Chapter 2, Appendix A).

The results of the two-way ANOVA were reported under each graph with F-values and degrees of freedom, and effects that are not significant are denoted “n.s.”. Significant results from the post-hoc Student-Newman-Keuls testing were annotated on the graphs itself, using asterisks for effect of acute stress (where significant differences between swim-stressed and non-stressed groups were denoted as *p<0.05, **p<0.01, ***p <0.001) and hashes for effect of prenatal stress (where significant differences between control and PNS groups were denoted as # p<0.05, ##p<0.01, ###p<0.001).

Individual data points were plotted, and outliers with two standard deviations away from the mean were removed, especially for baseline groups. Outliers always consisted of the same rat from corticosterone, DOC, DHDOC and THDOC measurements; the same individual rat from pregnenolone, progesterone, DHP and allopregnanolone measurements; whilst for testosterone, data from a separate rat was removed.

As an alternative way of analysing the dataset, three-way ANOVAs were additionally carried out where the male and female dataset were combined, which could potentially increase statistical power. Normality and variance were first visually examined by the plotting of residuals (Appendix A). Due to unequal variance between males and females, natural log transformation had to be carried out for all analytes except for corticosterone. However, it was difficult to present this data or do post-hoc testing due to the number of comparisons involved, therefore the results of the three-way ANOVA are included in Appendix C (Table C1) and only used to comment on the presence of sex differences.

Comparisons of concentrations between plasma and brain regions were also carried out using a two-way ANOVA, with results also attached in Appendix C (Table C2 and Table C3). Region (i.e. plasma vs brain region) and acute stress status are assessed as the two main factors, whilst post-hoc comparisons were carried out using Student-Newman-Keuls test, to compare plasma steroid concentrations versus steroid concentrations in the various brain regions. As 1 ml of plasma weighs close to 1 g of tissue (Taves et al., 2010), the absolute values for plasma concentrations (in ng/ml) and brain concentrations (in ng/g) were directly compared.

4.3 RESULTS

4.3.1 Corticosterone

Plasma: As expected, corticosterone secretion into the plasma was significantly greater in rats that were exposed to acute swim stress as compared rats which did not undergo swim stress in both males (Figure 4.5A) and females (Figure 4.5G), regardless of prenatal stress status. PNS offspring (both males and females) did not differ in plasma corticosterone concentrations as compared to controls, both basally and 30 min after acute swimming stress. There were no sex differences in plasma corticosterone concentrations between males and females (three-way ANOVA; Appendix C). Plasma corticosterone concentrations are also greater than concentrations in all the brain regions, both in males and females.

Male brain regions: In all brain regions of the male offspring, swim stressed groups had significantly greater corticosterone concentrations as compared to non-swim stressed groups (Fig 4.5B-F). Corticosterone concentrations did not differ between PNS and control rats in all brain regions investigated except for the male brainstem (Fig 4.5F), where corticosterone concentrations were significantly greater in swim-stressed PNS offspring as compared to swim-stressed control offspring.

Female brain regions: Swim-stressed groups had significantly greater corticosterone concentrations as compared to their respective non swim-stressed groups, in both control and PNS rats, in all female brain regions (Fig 4.5H-L). Corticosterone concentrations were not significantly different between control and PNS groups in all brain regions. A significant main effect of sex was also found in the frontal cortex, hypothalamus, and brainstem using a three-way ANOVA (Appendix C), indicating that corticosterone concentrations in these regions were generally greater in females than males in the respective treatment groups.

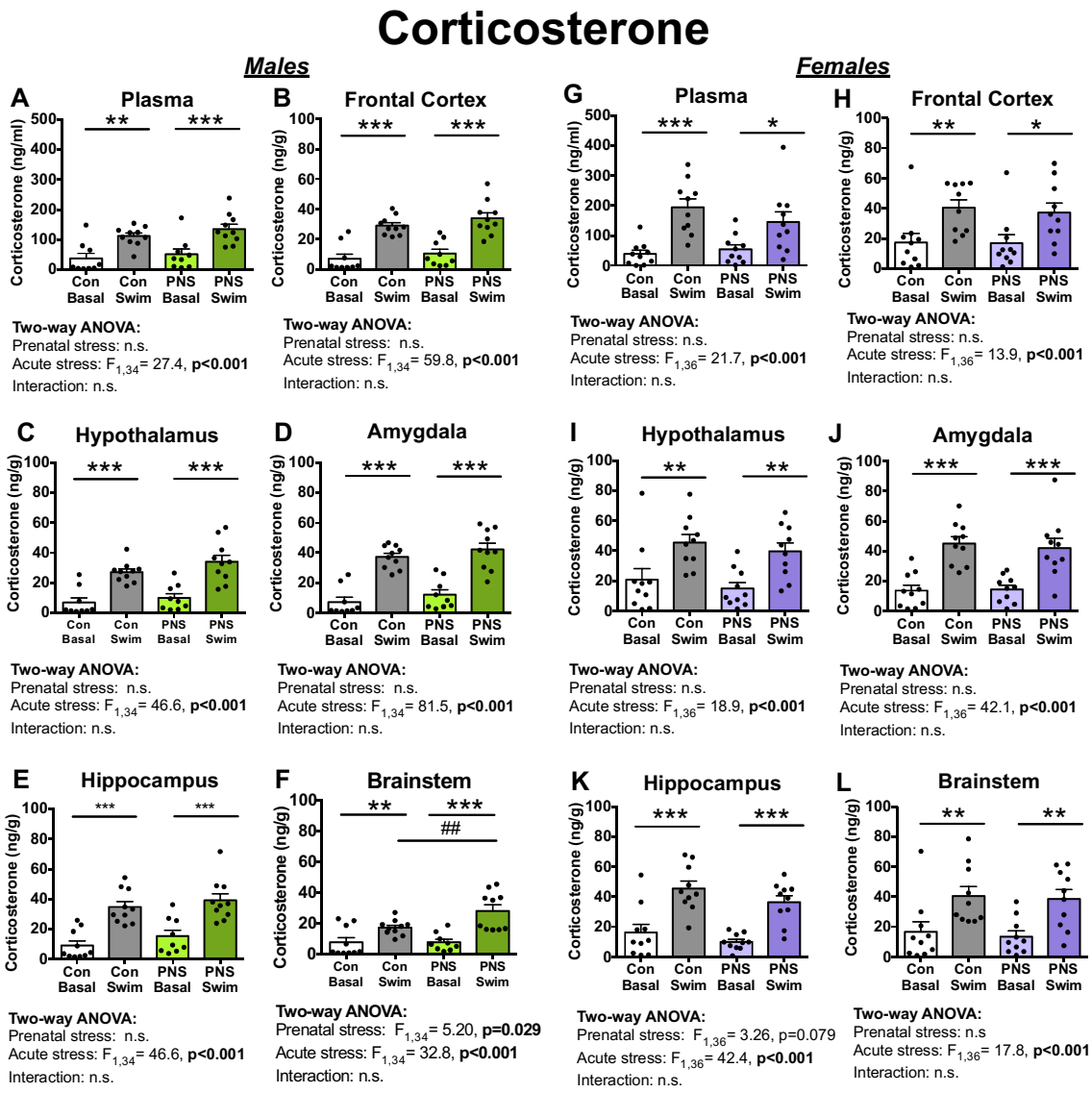


Figure 4.5: Corticosterone concentrations in the plasma and brain regions of male and female control (Con) and PNS offspring. Two-way ANOVAs were carried out separately for males and females, with F values and p-values reported below each graph. n.s.: not significant. A significant main effect of acute stress was observed in all regions investigated, for both males (A-F) and females (G-L). Post-hoc comparisons between basal and swim groups indicated that swim-stressed groups had significantly greater corticosterone concentrations in the plasma and all brain regions as compared to control groups (where *p<0.05, **p<0.01, ***p <0.001). There was an additional main effect of prenatal stress observed only in the male brainstem (F), where male PNS swim-stressed rats had greater brainstem corticosterone concentrations as compared to male control swim-stressed rats (where ## p<0.01). Due to higher concentrations of corticosterone in the plasma as compared to the brain, scale of Y-axes are different between plasma (A and G) and brain regions (B-F, H-L) in both sexes.

4.3.2 DOC, DHDOC and THDOC

Plasma: In males, DOC concentrations (Fig 4.6A) were significantly greater in the swim-stressed groups as compared to basal groups regardless of prenatal stress status. There were no differences in plasma concentrations of DHDOC (Fig 4.7A) and THDOC (Fig 4.8A) in males across all four groups. In the females, greater plasma concentrations of DOC (Fig 4.6G) and THDOC (Fig 4.8G) were detected in the swim-stressed females as compared to the basal group, regardless of prenatal stress status. There were no differences in plasma DHDOC concentration across all four female groups (Fig 4.7G). In the three-way ANOVA investigating sex effects (Appendix C, Table C1), a main effect of sex was observed for DOC, DHDOC and THDOC, where females had significantly greater concentrations as compared to males. DOC concentrations were not different between plasma and brain regions, whilst DHDOC and THDOC were slightly greater in the brain regions, in but only in certain brain regions like the brainstem, hippocampus and hypothalamus (Appendix C, Table C3).

Male brain regions:

DOC: Swim-stressed rats had significantly greater DOC concentations than non-swim stressed rats in all brain regions regardless of prenatal stress status, in most brain regions (Fig 4.6A-E), apart from the brainstem (Fig 4.6F). PNS and control groups also did not differ in the DOC concentrations in most of the brain regions (Fig 4.6A-E), apart from the brainstem (Fig 4.6F). In the brainstem (Fig 4.6F), only PNS but not control swim-stressed rats had greater DOC concentrations as compared to the respective non-swim stressed group. Additionally, PNS swim-stressed rats also had significantly greater brainstem DOC concentrations as compared to the swim-stressed control group (Fig 4.6F).

DHDOC: Swim-stressed rats had significantly greater DOC concentations than non-swim stressed rats regardless of prenatal stress status, in all brain regions except for the hypothalamus (Fig 4.7C). In the hypothalamus (Fig 4.7C), only control swim-stressed rats had significantly greater DHDOC concentrations as compared to the respective basal group, whilst the difference in the basal and swim-stressed PNS groups were not statistically significant ($p=0.063$). PNS and control groups did not differ in DHDOC concentrations in all brain regions.

THDOC: In the hypothalamus, THDOC concentrations were greater in swim-stressed groups as compared to non-swim stressed basal group, for both control and PNS male offspring (Fig 4.8D). In the frontal cortex, amygdala and brainstem, THDOC concentrations were significantly greater only in the control swim-stressed group as compared to control basal groups. In the PNS rats, differences between basal and swim-stressed PNS groups were not statistically significant (frontal cortex, p=0.06, brainstem, p=0.08, amygdala, p=0.20). There were no differences in THDOC concentrations in PNS and control groups in these brain regions. In the hippocampus (Fig 4.8E), there were no differences across all four groups.

Female brain regions:

DOC: Swim-stressed female rats had significantly greater DOC concentations than non-swim stressed rats in all brain regions regardless of prenatal stress status, in all brain regions (Fig 4.6H-L). Concentrations of DOC were not significantly different between control and PNS groups, in both basal and swim-stressed conditions, in most brain regions apart from the hippocampus (Fig 4.6I). In the hippocampus (Fig 4.6I), PNS swim-stressed females had lower concentrations of DOC as compared to the swim-stressed control group, indicating that PNS rats had a smaller DOC response following acute stress as compared to the controls. In all female brain regions, a main effect of sex was observed in the three-way ANOVA (Appendix C, Table C1), indicating that females had greater DOC concentrations in all brain regions as compared to males.

DHDOC: Swim-stressed female rats had significantly greater DOC concentations than non-swim stressed rats in all brain regions regardless of prenatal stress status, in most brain regions apart from the frontal cortex. In the frontal cortex (Fig 4.7H), control swim-stressed rats had significantly greater DHDOC concentrations as compared to control basal rats, however, in the PNS groups, swim-stressed and basal groups did not have significantly different DHDOC concentrations (p=0.0675). In all brain areas, PNS and control groups did not differ in DHDOC concentrations, in both the basal and swim-stressed conditions. In most brain areas apart from the hypothalamus, a main effect of sex was observed in the three-way ANOVA (Appendix C, Table C1), where females had greater DHDOC concentrations as compared to males except for the hypothalamus.

THDOC: Swim-stressed female rats had significantly greater THDOC concentations than non-swim stressed rats regardless of prenatal stress status in the hypothalamus, amygdala and the brainstem. In frontal cortex, only control swim-stressed females had greater THDOC concentrations than its respective basal group, whereas in the hippocampus, only PNS swim-stressed females had greater THDOC concentrations than the respective basal group. In all cases, THDOC concentrations in the PNS groups were not significantly different from the control group, both basally and after swimming stress. In most brain areas apart from the hypothalamus, a main effect of sex was observed in the three-way ANOVA (Appendix C, Table C1), where females had greater THDOC concentrations as compared to males except for the hypothalamus.

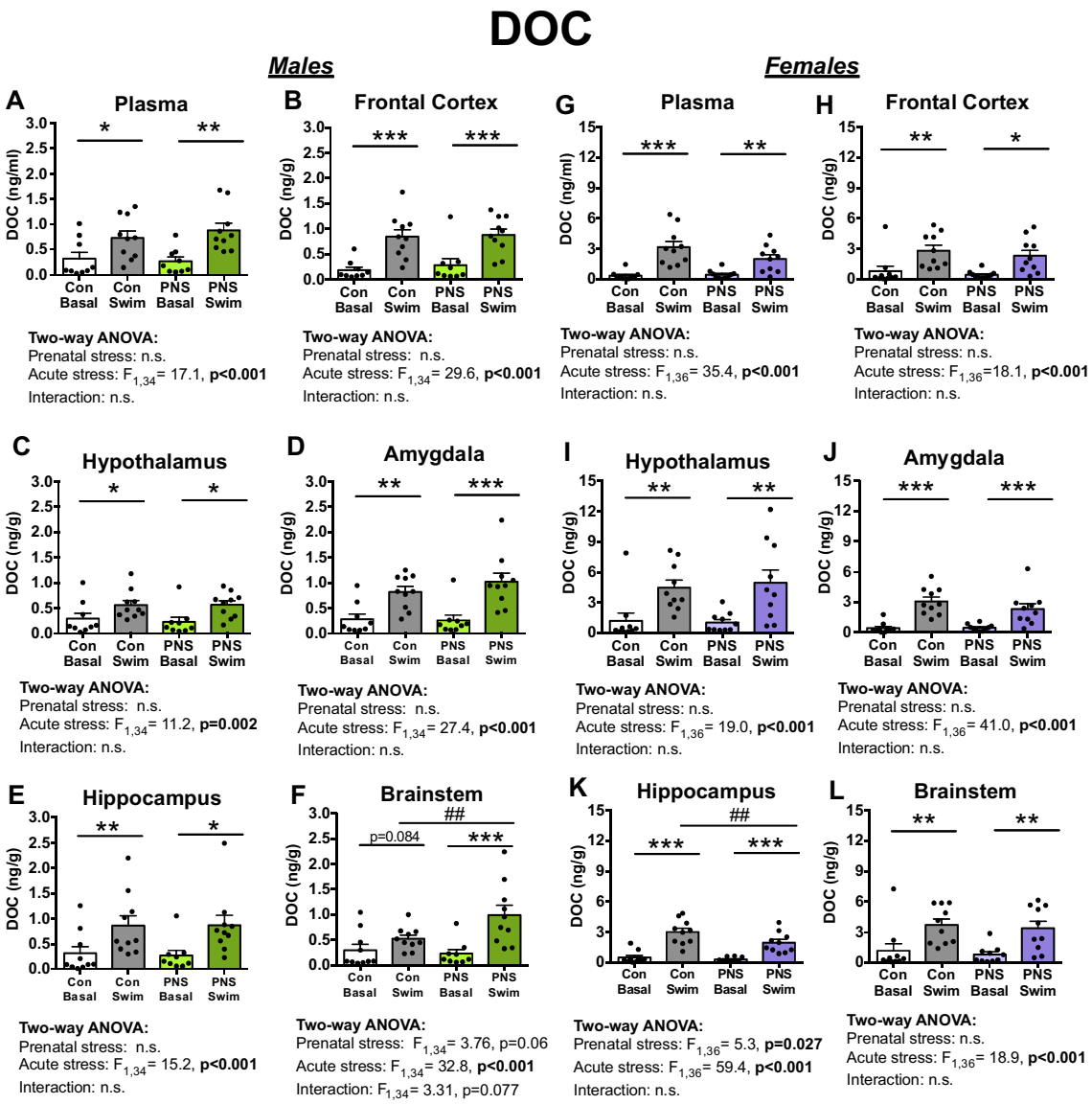


Figure 4.6: DOC concentrations in the plasma and brain regions of male and female control (Con) and PNS offspring. A significant main effect of acute stress was observed in all regions investigated, for both males (A-F) and females (G-L). Post-hoc comparisons revealed that all swim-stressed groups have significantly greater DOC concentrations when compared to control groups in the plasma and most brain regions (where * p<0.05, ** p<0.005, *** p <0.005), except for the male brainstem, where the difference between the control swim-stressed group and control basal group was not significant (p=0.084). Additionally, in the male brainstem, post-hoc testing also showed that PNS swim-stressed rats had signicantly greater DOC concentrations as compared to control swim-stressed rats (where ## p<0.01). A main effect of prenatal stress was also present for the female hippocampus (K), where female PNS swim-stressed rats had a lower hippocampal corticosterone concentration as compared to female control swim-stressed rats (where ## p<0.01). Due to a sex difference in the concentrations of DOC, range of Y-axes are different between males (A-F) and females (G-L). n.s.: not significant.

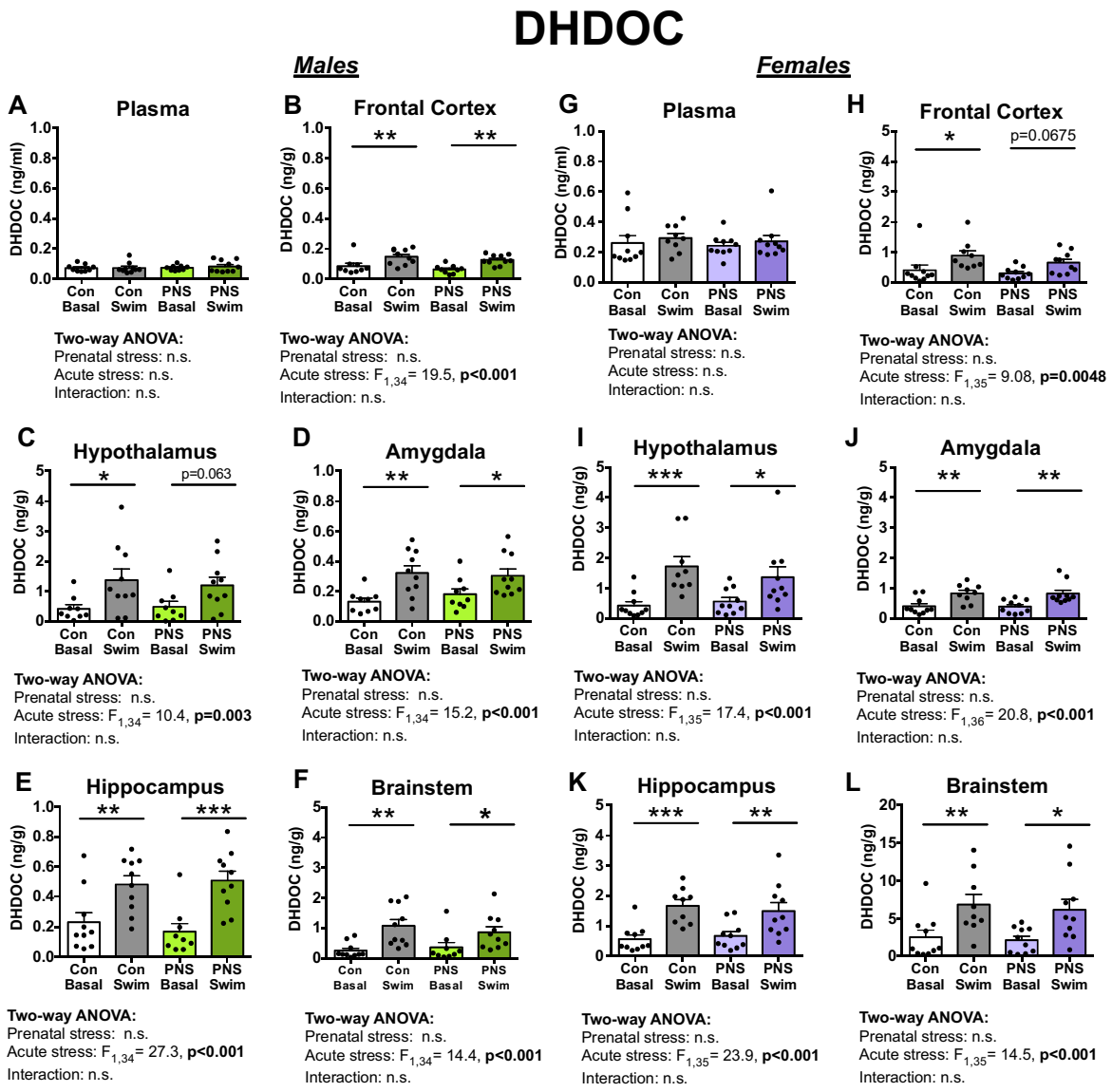


Figure 4.7: DHDOC concentrations in the plasma and brain regions of male and female control (Con) and PNS offspring. Two way ANOVAs were carried out separately for males and females, with F values and p-values reported below each graph. n.s.: not significant. There were no differences in all four groups for both male (A) and female plasma (G). Significant main effects of acute stress were observed in most brain regions investigated in both sexes, where swim-stressed rats had greater DOC concentrations than rats that did not undergo swim stress (*p<0.05, **p<0.01, ***p<0.001), except for the male hypothalamus (C) and female frontal cortex (H) where the differences between PNS basal and swim-stressed groups were not significant. There were no main effects of prenatal stress nor interactions, and controls and PNS rats did not have different basal DHDOC concentrations and did not respond differently to acute stress. Due to differences in the range of DHDOC concentrations in plasma and brain, as well as sex differences, range of Y-axes are different between plasma and various brain regions, notably the female brainstem (L). Females generally have greater DHDOC concentrations.

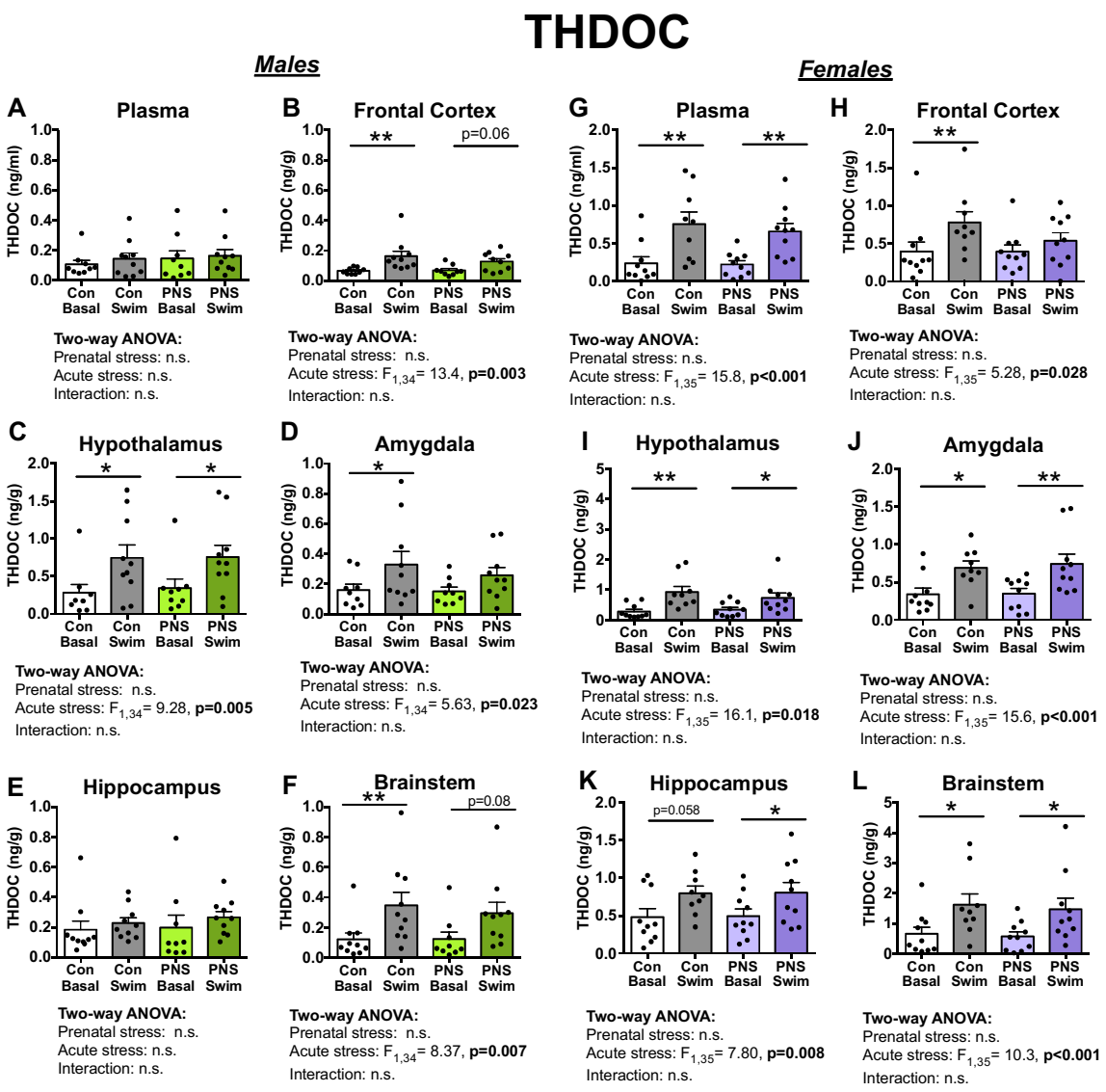


Figure 4.8: THDOC concentrations in the plasma and brain regions of male and female control (Con) and PNS offspring. A significant main effect of acute stress was observed in female plasma (G) but not in male plasma (A), where swim-stressed female groups had significantly greater THDOC concentrations as compared to basal groups (where **p<0.01). Significant main effects of acute stress were observed in most of the brain regions investigated, except for the male hippocampus where there were no differences across all four groups (E). Post-hoc testing revealed that swim-stressed groups had greater THDOC concentrations in both controls and PNS conditions in the male hypothalamus (C), female hypothalamus (I), female amygdala (J) and female brainstem (L), where *p<0.05 and **p<0.01. In the male frontal cortex (B), male amygdala (D), male brainstem (F) and female frontal cortex (H), differences between swim-stressed and control groups were only significant in the control groups, while in the female hippocampus (K), differences between swim-stressed and control groups were only significant in the PNS group. Note that the scale of Y-axes are different between the sexes, in the plasma and in different brain regions. Females generally have greater THDOC concentrations. n.s.: not significant.

4.3.3 Progesterone, DHP and allopregnanolone

Plasma:

Progesterone: In males, swim-stressed groups had significantly greater plasma progesterone concentrations as compared to basal groups, irrespective of prenatal stress status (Fig 4.9A). In females, a trend for significantly greater plasma progesterone concentrations was observed in the swim-stressed groups as compared to the basal groups, but the difference was not significant, in both control and PNS rats (Fig 4.9G). A main effect of sex was present in the three-way ANOVA, where females had greater plasma progesterone concentrations as compared to males. Progesterone concentrations were generally greater in the brain than in the plasma, except for the amygdala (both male and female), male frontal cortex and female hippocamus, where concentrations were similar (Appendix C, Table C3).

DHP: In males, control swim-stressed groups had significantly greater DHP plasma concentrations than control basal males, while PNS swim-stressed groups did not have greater concentrations as compared to the PNS basal group (Fig 4.10A). Additionally, following swimming stress, male control rats had significantly greater concentrations of plasma DHP as compared to male PNS rats. In females, there were no differences in plasma DHP concentrations across all four groups (Fig 4.10G). A main effect of sex was present in the three-way ANOVA, where females had greater plasma DHP concentrations as compared to males. DHP concentrations were generally greater in the brain except for the amygdala, frontal cortex, and female hippocampus, where concentrations were not different (Appendix C).

Allopregnanolone: In males, plasma allopregnanolone concentrations were greater in swim-stressed groups as compared to basal groups, regardless of prenatal stress status (Fig 4.11A). There were no differences across all four groups for plasma allopregnanolone concentrations in females (Fig 4.11G). A main effect of sex was present in the three-way ANOVA, where females had greater plasma allopregnanolone concentrations as compared to males. Allopregnanolone concentration were greater in the brain regions as compared to that in the plasma in males, however in the females, plasma concentrations were greater than that in the brain, except for the brainstem, where concentrations were not different as compared to plasma (Appendix C, Table C3).

Male brain regions:

In all male brain regions, concentrations of progesterone (Fig 4.9B-F), DHP (Fig 4.10B-F) and allopregnanolone (Fig 4.11B-F) were greater in swim-stressed rats as compared to the respective basal groups, irrespective of prenatal stress status. Concentrations of brain progesterone, DHP and allopregnanolone all did not differ significantly between PNS rats and control rats, both basally and after acute swimming stress, in all brain regions investigated. The three-way ANOVA (Appendix C, Table C3) revealed that there was a main effect of sex in all brain regions, indicating that males had lower brain progesterone, DHP and allopregnanolone concentrations as compared to females.

Female brain regions:

Progesterone: In the female brain, progesterone concentrations were significantly greater in PNS swim-stress groups as compared to the PNS basal groups only in the frontal cortex (Fig 4.9H) and the hypothalamus (Fig 4.9I). There were however no differences in progesterone concentrations observed between swim-stressed and basal progesterone levels in control groups in the frontal cortex and the hypothalamus. No significant differences across all four groups were observed in the amygdala, hippocampus and brainstem.

DHP: In the female frontal cortex (Fig 4.10H), swim-stressed PNS females had significantly greater DHP concentrations as compared to the basal PNS females. No significant differences were observed between the swim-stressed control group as compared to the basal control group in the frontal cortex. In all other brain regions (Fig 4.10I-L), there were no significant differences observed across all four groups.

Allopregnanolone: In the female frontal cortex (Fig 4.11H), hypothalamus (Fig 4.11I), and brainstem (Fig 4.11L), allopregnanolone concentrations were significantly greater in swim-stressed PNS females compared to the basal PNS females, however, no significant differences were observed between the control swim-stressed and the control basal groups. In the amygdala and hippocampus, no significant differences were observed across all four groups.

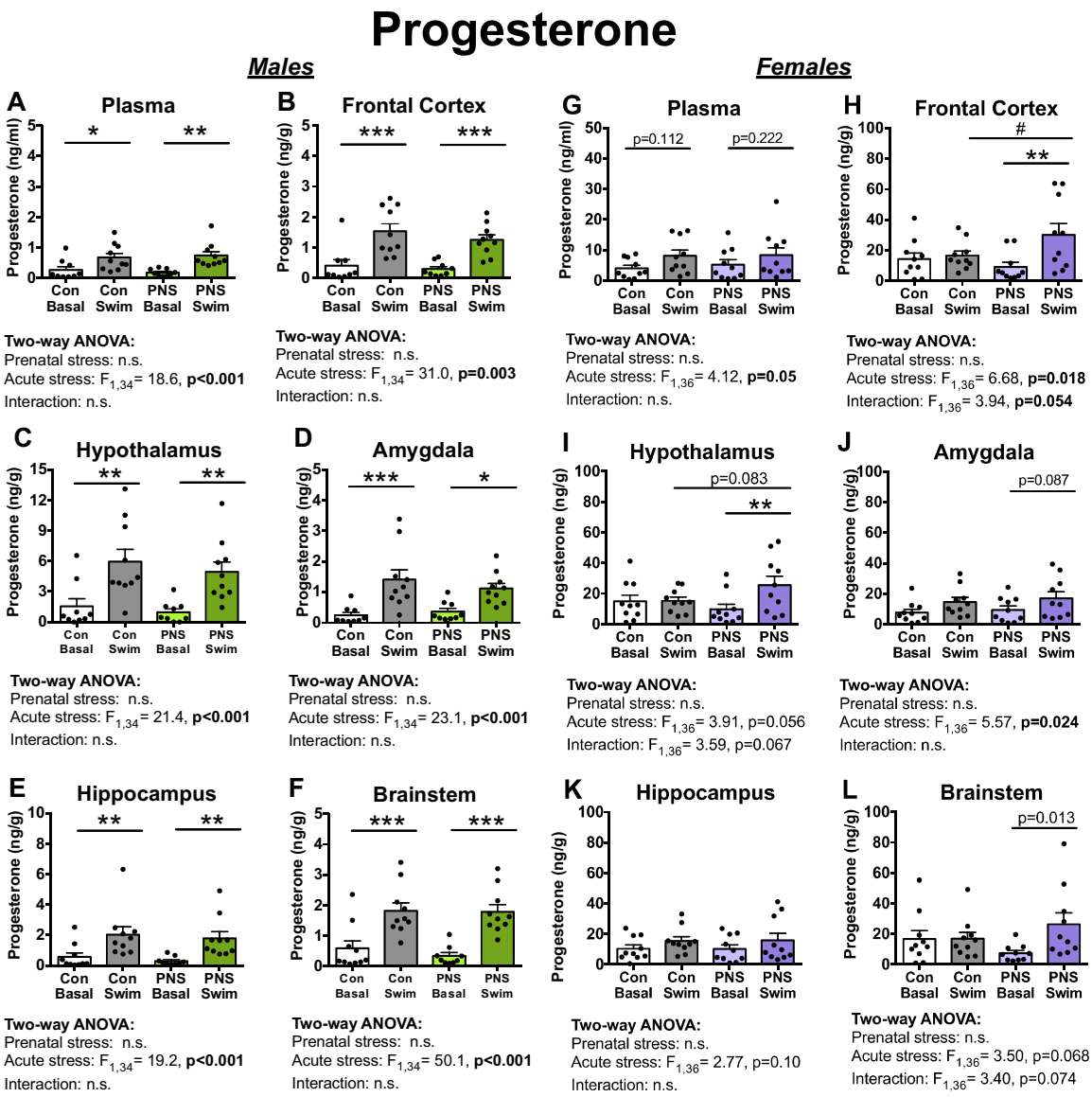


Figure 4.9: Progesterone concentrations in the plasma and brain regions of male and female control (Con) and PNS offspring. A significant main effect of acute stress was observed in plasma and all brain regions investigated in males (A-F), where *p<0.05, **p<0.01, ***p<0.001. In females, two-way ANOVA revealed that a main effect of acute stress was significant for the plasma (G) and some brain regions (H, J) but approaching significance for the others (I, K, L). Post-hoc testing revealed that in the frontal cortex (H), hypothalamus (I) and brainstem (L), only PNS but not control groups showed significant differences when comparing swim-stressed and basal conditions, whilst there were no differences in all four groups in the plasma (G), amygdala (J) and hippocampus (K). Additionally, in the female frontal cortex (H), an acute x prenatal stress interaction was present, and PNS swim-stressed rats had greater progesterone concentrations compared to control swim-stressed rats (where #p<0.05). n.s.: not significant.

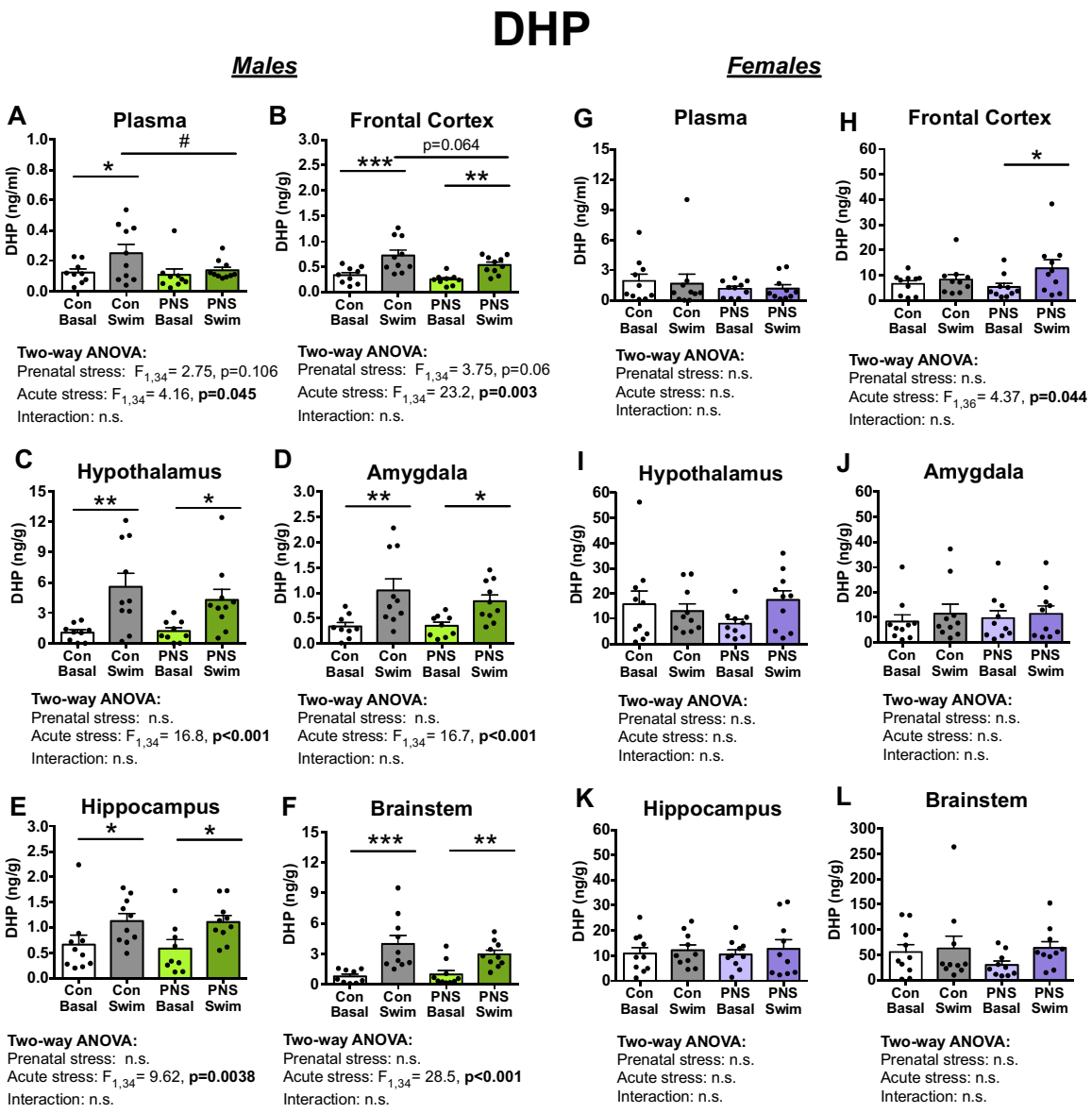


Figure 4.10: DHP concentrations in the plasma and brain regions of male and female control (Con) and PNS offspring. In the male plasma (A), a significant main effect of acute stress was observed, with additional effects of prenatal stress that was approaching significance. Post-hoc testing revealed that only control rats showed differences between swim-stressed and basal groups (where *p<0.05), and PNS swim-stressed group additionally had lower plasma DHP compared to control swim-stressed group (where # p<0.05). Significant main effects of acute stress were also observed in all brain regions investigated in males (B-F), where swim-stressed rats had greater concentrations of brain DHP as compared to basal groups in both control and PNS conditions (*p<0.05, **p<0.01, ***p<0.001). In females (G-L), a main effect of acute stress was only observed in the frontal cortex (H), where only PNS, but not control, swim-stressed rats had a greater concentration of DHP as compared to the respective basal group (*p<0.05). Note that the scale of Y-axes are different between the sexes, both in plasma and the different brain regions due to sex and regional differences in DHP concentrations. n.s.: not significant.

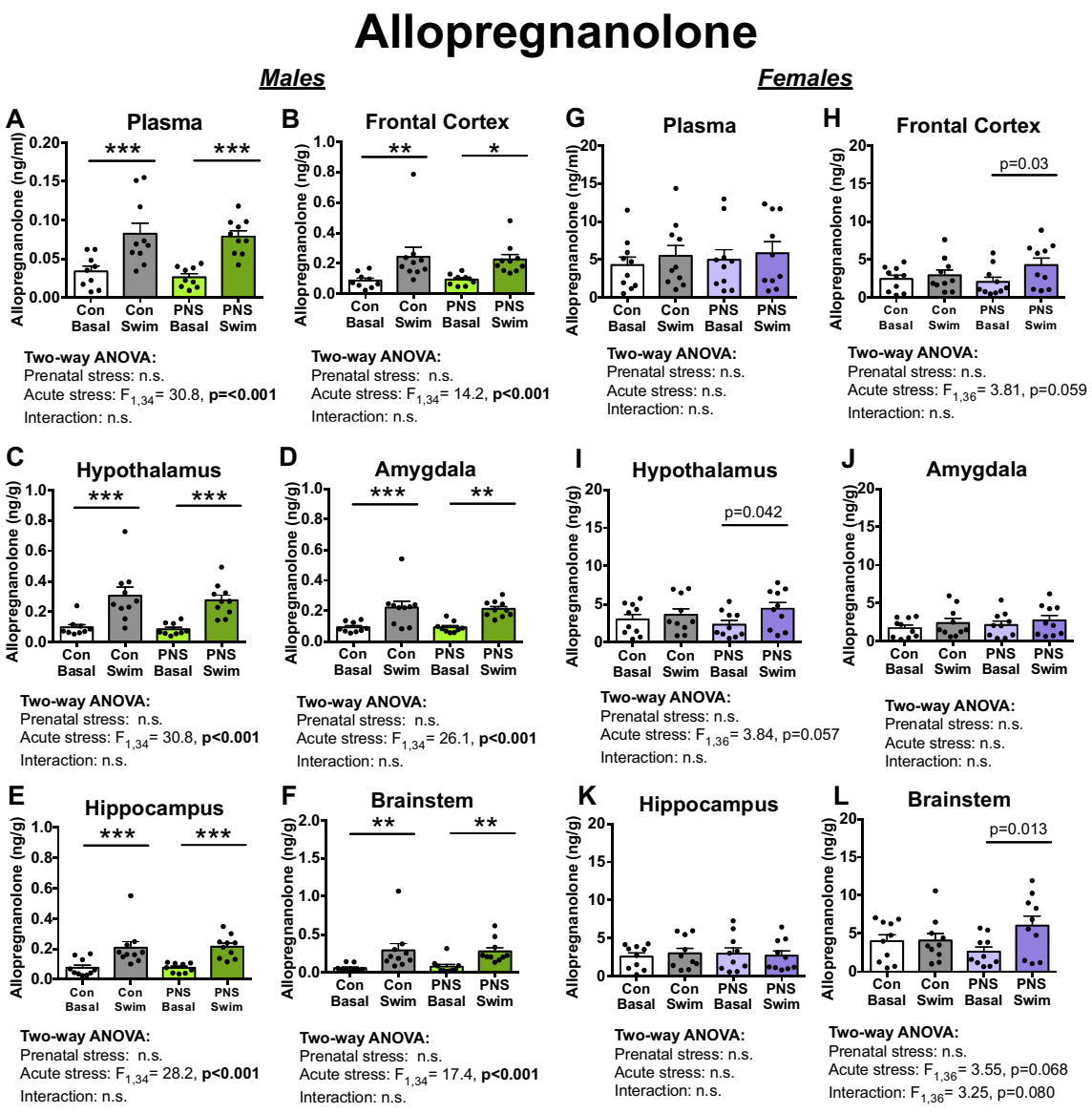


Figure 4.11: Allopregnanolone concentrations in the plasma and brain regions of male and female control (Con) and PNS offspring. A significant main effect of acute stress was observed in plasma and all brain regions investigated in males (A-F), where swim-stressed groups had greater allopregnanolone concentrations compared to the respective stressed groups (*p<0.05, **p<0.01, ***p<0.001). In females (G-L), two-way ANOVA revealed that a main effect of acute stress was approaching significance for the frontal cortex (H), hypothalamus (I), and brainstem (L), and a acute x prenatal stress interaction which was close to significance for female brainstem (L). In these three areas (H, I, L), post-hoc testing showed that the difference between swim-stressed and basal groups were only significant in the PNS rats but not control rats. In the female plasma (G), amygdala (J) and hippocampus (K), there were no differences in allopregnanolone concentrations across all four groups. n.s.: not significant.

4.3.4 Pregnenolone

Pregnenolone concentrations were significantly greater in all swim-stressed groups as compared to the respective basal groups in the plasma and in all of the brain regions, regardless of prenatal stress status and sex (Fig 4.12A-L). No significant differences in pregnenolone concentrations were observed between controls and PNS rats, both under basal conditions and following acute swim stress. A sex difference was observed (Appendix C) where females had greater absolute concentrations of pregnenolone as compared to males, in the plasma and in all the brain regions. Concentrations of all brain regions were greater than that found in the plasma, for both males and females (Appendix C, Table C3).

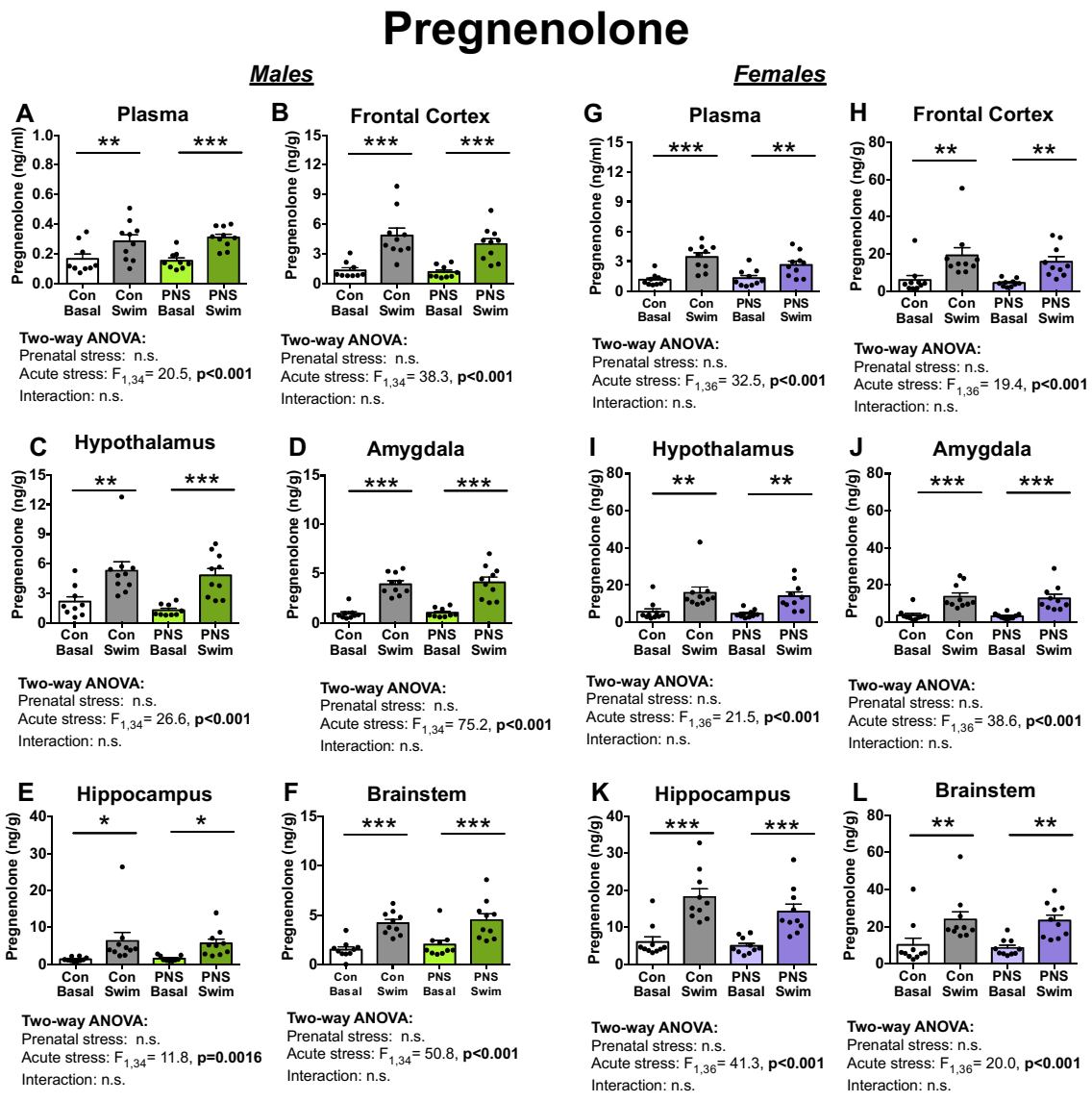


Figure 4.12: Pregnenolone concentrations in the plasma and brain regions of male and female control (Con) and PNS offspring. A significant main effect of acute stress was observed in plasma and all brain regions investigated in both sexes, where pregnenolone concentrations were greater in swim-stressed groups as compared to respective basal groups (*p<0.05, **p<0.01, ***p<0.001). There were no main effects of prenatal stress nor interactions, indicating that controls and PNS rats did not differ in plasma and brain pregnenolone concentrations. Scale of Y-axes are different between males and females, while the scale of plasma and hippocampus also differ from the rest of the brain regions. n.s.: not significant.

4.3.5 Testosterone

Testosterone was not significantly different between any of the four groups within each sex (Fig 4.13A-L). As expected, a sex difference was observed in the three-way ANOVA (Appendix C) where males had significantly greater concentrations of testosterone in the plasma and all brain regions investigated. Testosterone concentrations were similar in the plasma and in the brain, except for the male hippocampus, female brainstem and female hypothalamus, where brain concentrations were greater (Appendix C, Table C3).

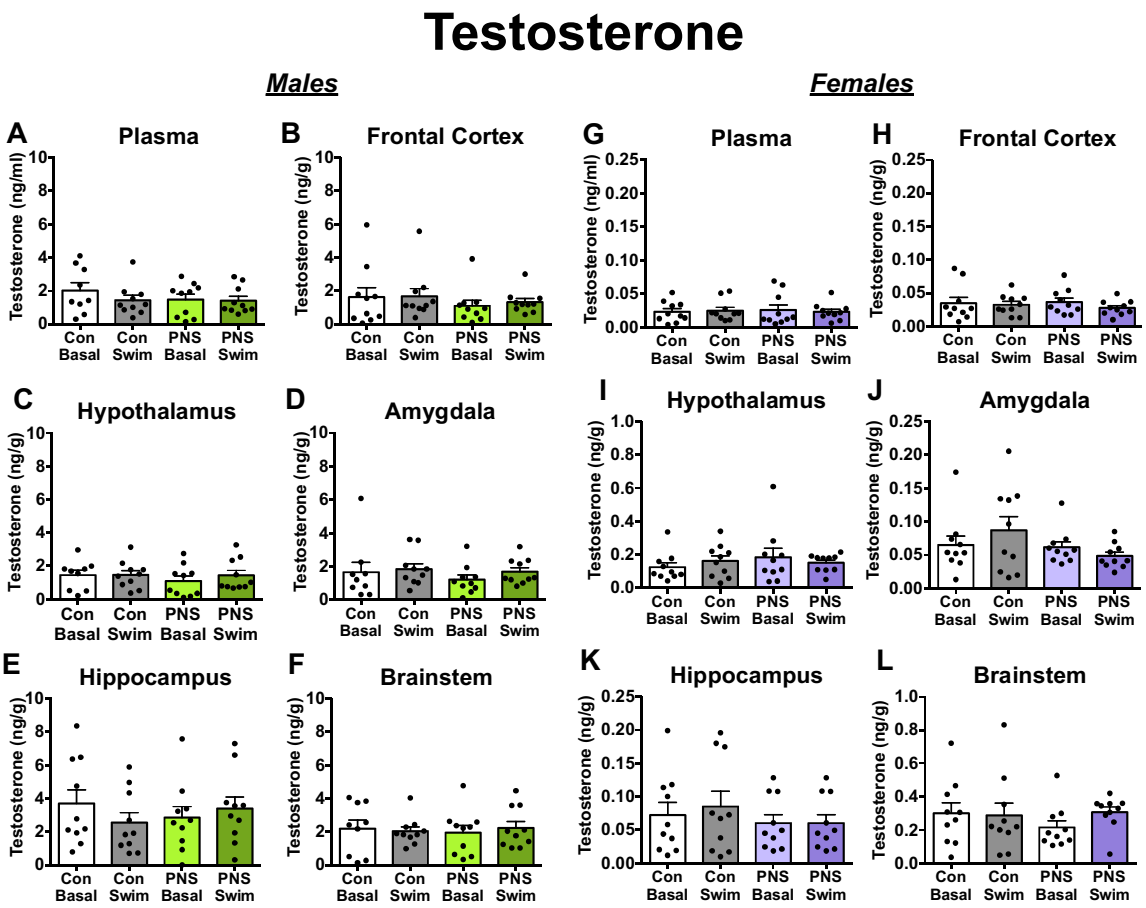


Figure 4.13: Testosterone concentrations in the plasma and brain regions of male and female control (Con) and PNS offspring. Two-way ANOVAs were carried out separately for males and females. There were no main effects of acute stress nor prenatal stress for both males (A-F) and females (G-L), and testosterone concentrations were not different across all four groups. Males have greater testosterone concentrations in the plasma and all brain regions, which is reflected in the difference in scale of the Y-axes.

4.3.6 Summary of results

The key findings in this chapter are summarised in the table below (Table 4.2). There were no differences in basal concentrations of any of the steroids investigated between control and PNS groups within each sex, in both plasma and in all of the brain regions examined. As expected (Sze et al., 2018), an effect of acute stress was noted for most neuroactive steroids in the brain, where the swim-stressed groups had significantly greater concentrations than the non-stressed groups. Notably, differences between the PNS and control groups were present in some cases with acute swim stress, where PNS swim-stressed groups seemed to have either greater or lower concentrations as compared to the control swim-stressed groups within each sex. These differences seemed to be sex-dependent (i.e. observed in one sex but not the other), and brain region-dependent (i.e. observed in some regions but not in the others).

Steroid/ Region	Male	Female
Plasma corticosterone	• Acute stress resulted in greater corticosterone concentrations • PNS and control are not different	• Acute stress resulted in greater corticosterone concentrations • PNS and control are not different
Brain corticosterone	• Acute stress resulted in greater corticosterone concentrations in all brain regions • PNS > Control only in acute stress conditions in brainstem • PNS and controls were not different in other brain regions	• Acute stress resulted in greater corticosterone concentrations in all brain regions • PNS and controls were not different
Plasma DOC	• Acute stress resulted in greater DOC concentrations • PNS and controls were not different	• Acute stress resulted in greater DOC concentrations • PNS and controls were not different
Brain DOC	• Acute stress resulted in greater DOC concentrations in all brain regions except for the brainstem • Acute stress resulted in greater DOC concentrations only in PNS group in the brainstem • PNS > Control only in acute stress conditions in brainstem • PNS and controls were not different in other brain regions	• Acute stress resulted in greater DOC concentrations in all brain regions • PNS < Control only in acute stress conditions in hippocampus • PNS and controls were not different in other brain regions

Plasma DHDOC	• Acute stress did not change plasma DHDOC concentrations • No differences between PNS and controls	• Acute stress did not change plasma DHDOC concentrations • No differences between PNS and controls
Brain DHDOC	• Acute stress resulted in greater DHDOC concentrations in all brain regions except for PNS group in hypothalamus • No differences between PNS and controls	• Acute stress resulted in greater DHDOC concentrations in all brain regions except for PNS group in FC • No differences between PNS and controls
Plasma THDOC	• Acute stress did not change plasma THDOC concentrations • No differences between PNS and controls	• Acute stress resulted in greater plasma THDOC concentrations • No differences between PNS and controls
Brain THDOC	• Acute stress resulted in greater THDOC concentrations in both PNS and control in hypothalamus • Acute stress resulted in greater THDOC concentrations in controls but not PNS rats in FC, amygdala and brainstem • Acute stress did not alter THDOC concentrations in the hippocampus • No significant differences between PNS and controls	• Acute stress resulted in greater THDOC concentrations in both PNS and control in hypothalamus, amygdala and brainstem • Acute stress resulted in greater THDOC concentrations in controls but not PNS rats in FC • Acute stress resulted in greater THDOC concentrations in PNS but not control rats in hippocampus • No significant differences between PNS and controls
Plasma Progesterone	• Acute stress resulted in greater plasma progesterone concentrations • PNS and controls were not different	• Acute stress did not alter plasma progesterone concentrations • PNS and controls were not different
Brain Progesterone	• Acute stress resulted in progesterone concentrations in all brain regions • PNS and controls were not different	• Acute stress resulted in greater progesterone concentrations in PNS but not control rats in FC, hypothalamus and brainstem • Acute stress did not alter progesterone concentrations in the hippocampus and amygdala

		• PNS > Controls only in acute stress condition in FC • PNS and controls were not different in other brain regions
Plasma DHP	• Acute stress resulted in greater plasma DHP concentrations in controls but not PNS rats • PNS < Controls only in swim-stress condition	• Acute stress did not alter plasma DHP concentrations • PNS and controls were not different
Brain DHP	• Acute stress resulted in greater DHP concentrations in all brain regions • PNS and controls were not different	• Acute stress resulted in greater DHP concentrations in PNS but not control rats in FC • Acute stress did not alter DHP concentrations in all other brain regions • PNS and controls were not different
Plasma allopregnanolone	• Acute stress resulted in greater plasma allopregnanolone concentrations • PNS and controls were not different	• Acute stress did not alter plasma allopregnanolone concentrations • PNS and controls were not different
Brain allopregnanolone	• Acute stress resulted in greater allopregnanolone concentrations in all brain regions • PNS and controls were not different	• Acute stress resulted in greater allopregnanolone concentrations in PNS but not control rats in FC, hypothalamus, brainstem • Acute stress did not alter allopregnanolone concentrations in the amygdala and hippocampus • PNS and controls were not different
Plasma and brain pregnenolone	• Acute stress resulted in greater pregnenolone concentrations • PNS and controls were not different	• Acute stress resulted in greater pregnenolone concentrations • PNS and controls were not different
Plasma and brain testosterone	• Acute stress did not alter testosterone concentrations • PNS and controls were not different	• Acute stress did not alter testosterone concentrations • PNS and controls were not different

Table 4.2: Summary of the main observations in this study. A summary of whether swim stress altered concentrations of neuroactive steroids in the brain and plasma, and if PNS rats had different concentrations of neuroactive steroids as compared to controls. FC: Frontal cortex

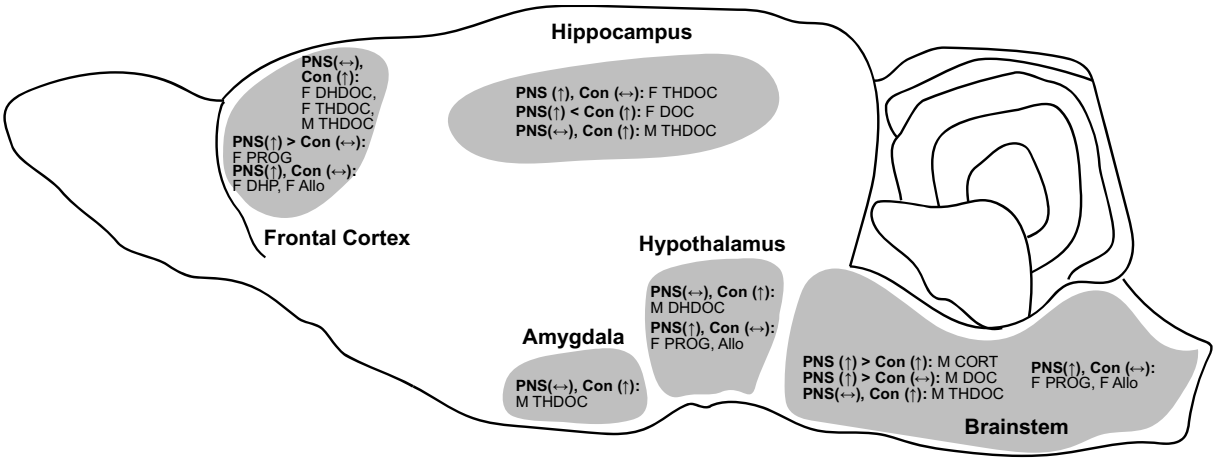


Fig 4.14 Pictorial representation of the summary of differences between PNS and control offspring. Arrows indicate if swim-stressed offspring have increased neuroactive steroid concentrations as compared to the respective non-stressed offspring, with (↑) representing greater concentrations with swim stress, whilst (↔) represents no significant differences between swim-stressed and basal groups. As for the comparison between control and PNS swim-stressed groups, (>) indicates significantly greater steroid concentrations in PNS groups as compared to control swim stress, whilst (<) indicates PNS groups having significantly lower steroid concentrations compared controls, and (≈) represents no significance differences between PNS and control swim-stressed groups. For steroids that are not mentioned in this diagram, controls and PNS did not respond differently with swim stress (i.e. both PNS and controls are either (↑) or (↔)), and steroid concentrations are also not different between PNS and controls (≈). F: Female, M: Male, ALLO: Allopregnanolone, PROG: Progesterone, CORT: Corticosterone. Figure is a representation of the different brain regions and does not anatomically represent any particular sagittal plane.

4.4 DISCUSSION

Three main conclusions emerge from these results, based on the hypotheses mentioned in section 4.1.4. (i) Firstly, PNS swim-stressed rats did not exhibit hypersecretion of corticosterone into the circulation when compared to the control swim-stressed group (section 4.4.1). Nonetheless, there were alterations in glucocorticoid concentrations in certain brain regions (notably, the male brainstem) of these PNS swim-stressed rats as compared to the control swim-stressed rats (section 4.4.2). (ii) Secondly, across the board, neuroactive steroid concentrations were not obviously different between control and PNS offspring, whether or not acute stress was given. There were no differences in the concentrations of neuroactive steroids under basal conditions between control and PNS offspring. However, there was some evidence that stress-induced concentrations of 5α- and 3α-reduced steroids, such as DHP and THDOC were lower in the PNS rats as compared to the controls, and this seemed to occur in more brain areas in the male offspring (section 4.4.3). Female data was however, may be confounded by oestrous cycle changes that were not considered in this study (section 4.4.4). (iii) Lastly, although there were no stark differences between PNS and controls in terms of neuroactive steroid concentrations within each sex and region, sex differences (section 4.4.5) and regional differences (section 4.4.6) were still observed, which may have functional implications.

4.4.1 HPA axis activity in PNS rats following acute swim stress

PNS offspring did not result exhibit an exaggerated plasma corticosterone response as compared to controls following swim stress, contrary to the hypothesis (section 4.1.4, point 1) and to previous studes, where there is greater plasma corticosterone observed in response to systemic IL-1β administration and restraint stress in PNS offspring exposed to the same prenatal social stress (Brunton et al., 2015, Brunton and Russell, 2010).

Nature of different stressors:

The observations in this study can most likely be attributed to different stressor types activating the HPA axis to different extents. IL-1β administration is a physical stressor, restraint is a psychogenic stressor, whilst swim stress has both physical and psychogenic components (Briski, 1996, Sawchenko et al., 2000, Dayas et al., 2001). The neural pathways associated with systemic IL-1β signalling to the PVN

have been elucidated in studies carried out in rats, where brainstem A2 NTS neurones activate CRH neurones in the PVN directly via a noradrenergic input (Cunningham and Sawchenko, 1988, Sawchenko et al., 2000).

The neural pathways activated by swim stress however, are more complex and have not been fully elucidated, but may involve brainstem noradrenergic signals (Douglas et al., 2005) and input from limbic structures (Duncan et al., 1996). In addition, stress signals from acute swim stress could have also been transmitted through a serotonergic pathway that originates from GABA neurones in the raphe nuclei of the brainstem (Roche et al., 2003).

Although both acute swim stress and restraint stress have psychogenic components, it is likely that differences still exist between these stressors. Swim stress can be considered a forced exercise, which could have metabolic consequences such as changes in blood pH, lactate, and/or CO_2 concentration (Abel, 1994). Although swim stress and restraint stress activate similar regions in the brain, including the limbic regions (Cullinan et al., 1995), there is evidence that these two stressors result in differential cellular outcomes, which could suggest activation of different neural circuits in the brain. For instance, differential patterns of phosphorylation of mitogen-activated protein kinases (MAPKs) are observed after a 15 min swim stress versus 30 min restraint stress in various different brain regions, possibly due to differences in NMDA receptor activation (Shen et al., 2004). In support of the complicated neural pathways that swim stress may induce, it was additionally found that behaviour during the swimming bout does not correlate with serum corticosterone levels (Marti and Armario, 1996). After all, the stress response is multifaceted, and individual variations in coping styles in response to a psychogenic stressor is likely to further complicate the final neuroendocrine output (Koolhaas et al., 2010).

Technical differences:

Additionally, there were several differences in terms of experimental set-up and blood sampling protocols, which makes direct comparison of this study and previous experiments problematic.

Firstly, in this experiment, the comparisons between non-stressed and acutely stressed steroid profile were carried out in different groups of rats. This was in

contrast with the previous blood sampling experiments, where blood was taken from the same rat, enabling one to measure pre-stress and post-stress corticosterone concentrations. Given the individual differences in steroid concentrations that are present, especially with the added influence of acute and prenatal stress (Williams, 2008, Boersma and Tamashiro, 2015, Koolhaas et al., 2010), it may be more difficult to detect differences between PNS and control groups with this experimental set-up. Secondly, here, plasma corticosterone data was only obtained at a single time point (30 min after the induction of 2 min swim stress), thus conclusions were unable to be drawn for whether there was a difference in the amplitude of the response in PNS rats or whether the corticosterone response to stress was prolonged in PNS offspring which is another way in which HPA axis dysregulation can manifest (i.e. area under curve of the cumulative corticosterone response). In addition, the acute swim stress paradigm here lasted for only 2 minutes, and at the time of blood collection, the stressor has ceased. This is in contrast with the restraint stress paradigm, where at 30 min after stress induction, rats were still undergoing restraint. The clearance of intravenously administered IL-1β also takes longer than 30 min (Klapproth et al., 1989). Hence, as well as the nature of the stressors being different, differences in the duration of the stressor could also contribute to the different neuroendocrine responses in the PNS offspring.

Moreover, in this experiment, rats were killed by conscious decapitation and trunk blood was collected. Although the collection of trunk blood versus venous blood should not make a huge difference in the quantification of circulating corticosterone concentrations, the rats in this experiment were naïve and did not undergo cannulation surgery before undergoing acute stress, in contrast with previous experiments. Cannulation surgery, although carried out four days before blood sampling, could be considered an additional chronic stressor to the rats (Vahl et al., 2005), and it is possible that the immune status of rats could possibly have been altered in the blood sampling experiment (DeKeyser et al., 2000). In these previous experiments, the cannulation surgery therefore could have contributed to the allostatic load, and the administration of the acute stressor (e.g. restraint or IL-1β administration) could then be considered as an additional stressor on top of the surgical stress, thereby augmenting the differences in corticosterone secretion between PNS and control offspring.

4.4.2 Glucocorticoids in the brain

Although there were no differences in plasma corticosterone concentrations between PNS and control offspring, brain corticosterone concentrations could still be different. Here, there were no differences in basal concentrations of corticosterone between control and PNS offspring in either sex in any of the brain regions, however, in the swim-stressed groups, there were instances where the PNS offspring had altered concentrations of corticosterone and DOC in certain brain regions as compared to controls, and these differences seem to be sex-dependent, suggesting that GR signalling in the brain following stress may be different in control and PNS rats.

Firstly, male swim-stressed PNS offspring had significantly greater concentrations of corticosterone and DOC as compared to the control swim-stressed group. This observation was specific for the male brainstem, and was not observed in other brain regions in the males, nor in the brainstem of the females. This observation could be a result of different rate and extent of uptake of glucocorticoids, depending on brain region and sex. In control rats, differences in the local uptake of corticosterone in different brain regions has indeed been reported (McEwen et al., 1969). In addition, a study which intravenously injected radioactively-labelled DOC into rats showed that the highest concentration of radioactive label corresponded to the reticular formation in the brainstem (Kraulis et al., 1975), attesting to the differential uptake of DOC in different brain regions. Whether or not the uptake process was altered in the PNS males as compared to control males and/or females remains unclear. Additionally, it is not known if the enzymes 11β-HSD1, 11β-HSD2, and 11β-hydroxylase (Fig 1.3) are affected in PNS offspring, as these enzymes can also control local corticosterone concentrations.

The functional significance of higher brainstem corticosterone and DOC concentrations in the brainstem of PNS male offspring following acute stress is also unclear, although glucocorticoid and noradrenergic pathways can interact to result in various coping and adaptation behaviours following stress (Krugers et al., 2012). Corticosterone administration in rats has been shown to alter noradrenergic metabolism in the locus coeruleus of the brainstem, upregulating the expression of the noradrenaline transporter, which has a role in noradrenaline uptake and controlling noradrenergic synaptic transmission (Fan et al., 2014). However, given

the complex interactions involved in the neural circuitry of stress signalling and the indirect innervations that the brainstem has with other brain regions, functional implications of altered corticosterone and DOC concentrations here remain only speculative.

In females, concentrations of DOC also seem to be affected by prenatal stress. However, in contrast to the males, the brain region affected was different, differences were also observed in the direction of change. DOC concentrations was lower in the hippocampus in the PNS swim-stressed group compared to the control swim-stressed group. Like the males, this observation could be a result of differential glucocorticoid uptake in PNS versus control offspring. Stress, in the form of social crowding and social isolation, has previously been shown to alter corticosterone uptake in the hippocampus of mice (Valeri et al., 1978), however, it is not known if prenatal stress will affect the hippocampus of the PNS female rats in the same manner. In terms of functional significance, it can be postulated that such a decrease could represent disrupted negative feedback inhibition on the HPA axis. Additionally, corticosterone in the hippocampus may be involved in the rapid non-genomic modulation of dendritic spines (Komatsuzaki et al., 2012, Murakami et al., 2018), thus a decrease in corticosterone concentrations may impact synaptic plasticity and therefore other behaviours (e.g. learning and memory related activities) in the PNS female.

4.4.3 5α- and 3α- reduced neuroactive steroids in PNS versus control rats

Although there was no hypersecretion of corticosterone into the circulation following acute swimming stress (as discussed in section 4.4.1) in PNS rats as compared to controls, concentration GABA modulatory steroids were still compared between controls and PNS, especially since mRNA expression of 5α-reductase type 1 is known to be altered in PNS offspring (Brunton et al., 2015). In general, in terms of 5α- and 3α,5α-reduced steroids (DHP, allopregnanolone, DHDOC, and THDOC), there were no obvious differences in concentrations of steroids when PNS and control animals are compared, under both basal and acute stress conditions, except for plasma DHP in PNS males. However, in females, DHP and allopregnanolone did not increase following acute stress in the control groups, possibly due to oestrous cycle variations, which may additionally confound the interpretation of the results in the PNS groups, which will be explained in the next section (section 4.4.4). Again,

these results are contrary to the hypothesis (section 4.1.4, point 2), however, these may be confounded by the fact there was no HPA axis hyperactivity as mentioned earlier.

In males, concentrations of plasma DHP were not increased in the PNS groups following swim stress, and additionally, swim-stressed PNS males had lower plasma DHP concentrations than that of the swim-stressed control group. The lower concentrations of peripheral DHP following stress in PNS males may imply that the production of DHP from the adrenal gland or liver is affected. Although 5α-reductase activity in the adrenal gland following early life insults have thus far not been investigated, there is evidence that hepatic 5α-reductase isoform 1 is downregulated as a result of prenatal programming (Brunton et al., 2013). In the brain, male PNS rats did not differ in DHP concentrations as compared to controls. Although it was also previously shown that 5α-reductase 1 mRNA expression was increased in the mPFC of PNS male rats (Brunton et al., 2015), the results here indicated that the mRNA changes did not affect DHP production in the brain, both basally and after swimming stress. For allopregnanolone, there was also no evidence that brain production of allopreganolone is compromised in the PNS male offspring, both basally and following swimming stress.

Conversely, for THDOC, the other neuroactive steroid that potentiates GABA signalling, although concentrations in the PNS groups were not statistically different from their respective controls, when comparing within the PNS groups, swim-stressed PNS rats did not have statistically greater concentrations of THDOC as compared to their non-swim stressed counterparts. This observation occurred in the male frontal cortex, amygdala and brainstem and the female frontal cortex. This could imply decreased THDOC generation and hence signalling, however, the functional implications of this are again unknown. It is tempting to suggest that inhibitory signalling in PNS offspring may be affected, impacting the regulation of the HPA axis at multiple levels, however, since allopregnanolone and THDOC binds to the same receptor, it is possible that allopregnanolone can compensate for the deficits in the production of THDOC. Nevertheless, it is known from electrophysiology studies in rats that THDOC and allopregnanolone can alter synaptic inhibition differently (Schwabe et al., 2005), probably due their differential potencies in modulating $GABA_A$ receptors of different subunit compositions. More studies will need to be carried out to determine if these differences have

physiological implications. Additionally, the fact that more brain regions in males were affected could point to the increased tendencies for such deficits in THDOC production in males as compared to females. In fact, differences were reported in the maximal $GABA_A$ receptor potentiation between male and female rats for THDOC (Wilson and Biscardi, 1997), attesting to sex differences in THDOC regulation.

The conclusion for this section is that concentrations of neuroactive steroids were not drastically impacted in PNS offspring, but there may be modest deficits in THDOC production after swim stress. Nevertheless, it is worthwhile highlighting again that in this experiment, contrary to the hypothesis, HPA axis hyperactivity was not clearly observed following stress in the PNS offspring, which may relate to the lack of differences in concentrations GABAergic neuroactive steroids between control and PNS offspring.

4.4.4 Changes in progesterone and its metabolites in females

Whilst progesterone and its metabolites were greater following swim stress in the male control offspring, in plasma and all the brain regions investigated, swim stress did not result in an increase in progesterone, DHP nor allopregnanolone concentrations in the control females, in both plasma and the brain regions. This is contrary to the hypothesis and in contrast to observations in previous studies, where progesterone, DHP and allopreganolone were increased in control swim-stressed animals regardless of sex (Sze et al., 2018).

The female data might have been confounded by oestrous cycle variations, which was not accounted for in either study. Although previous studies have determined that there is very little variation in brain concentrations of pregnenolone, progesterone, DHP, or allopregnanolone across the oestrous cycle in female mice (Corpechot et al., 1997), and that corticosterone secretion is not affected by oestrous cycle in female rats (Guo et al., 1994, Ogle and Kitay, 1977), plasma progesterone concentrations do vary across different stages of the oestrous cycle. Higher levels of progesterone occur twice in the 4-day oestrous cycle, with one peak between metoestrus and dioestrus, and another pre-ovulatory peak during pro-oestrus (Butcher et al., 1974). Additionally, although basal levels of corticosterone are not known to be affected by oestrous cycle changes, stress-induced secretion of ACTH and corticosterone can be affected by oestrous cycle changes, and are

highest when oestradiol levels were elevated (e.g. in pro-oestrus) (Viau and Meaney, 1991).

Additionally, the previous study (Sze et al., 2018) was also carried out in adult rats that were aged 21 weeks old, while this study was carried out in rats that were 7 weeks old (ca. 50 days old). Age therefore, could be a factor that affected progesterone production following stress. Although male and female rats reach sexual maturity at around 48 days of age and 34 days of age, respectively (Lewis et al., 2002), and HPA axis maturation is considered to be largely complete by that age (Evuarherhe et al., 2009), some studies have argued that that the period between PND 50 - 60 represents the grey zone between adolescence and adulthood, and some neurobehavioural responses may not have reached adulthood patterns yet (Spear, 2000).

Therefore, whilst the PNS female group largely did not differ from the control females in terms of progesterone and its metabolites, except in the frontal cortex, hypothalamus and brainstem, where a trend towards increased allopregnanolone was observed, the female controls did not seem to mount a neurosteroid response as reported in previous studies (Hueston and Deak, 2014, Sze et al., 2018). Further experiments with oestrous cycle and age taken into consideration may need to be carried out in order for the results observed in this study to be generalised to all PNS female offspring.

4.4.5 Sex differences in neuroactive steroid concentrations

Studies involving the direct measurement of male and female concentrations of neuroactive steroids in the same study have been limited, and to our knowledge, this is the third study, following our previous study (Sze et al., 2018), and a study by (Caruso et al., 2013), that has measured male and female neuroactive steroid levels in Sprague-Dawley rats in the same experiment. Absolute concentrations of neuroactive steroids were markedly different between males and females. Females had greater concentrations of DOC, DHDOC, THDOC, progesterone, DHP, allopregnanolone and pregnenolone as compared to males in plasma and in the various brain regions. In this study, whilst females did not have greater plasma corticosterone concentrations as compared to males, greater corticosterone concentrations in females was observed for the hypothalamus, frontal cortex and

brainstem. Testosterone was also more than ten-fold greater in the male plasma and brain regions as compared to that in the females.

Other than differences in absolute concentrations of neuroactive steroids, there were a few instances where the pattern of difference between controls and PNS were different between males and females. As discussed in section 4.4.2, corticosterone and DOC were greater in PNS swim-stressed male brainstem as compared to control swim-stressed group, but lower in PNS swim-stressed female hippocampus as compared to its respective control swim-stressed group. It thus seems that here, the manner by which steroid concentrations changed in PNS rats when compared to the controls is dependent on sex and brain region. In section 4.4.3, THDOC concentrations following stress were also more affected in the male brain as compared to the female brain, where three regions were affected in males, whilst for the female brain, only the frontal cortex was affected. Whilst the functional significance of these differences cannot be determined here, the presence of differences demonstrate the importance of taking both sexes into consideration when quantifying steroids.

Although it has been previously reported that prenatal stress in rats can de-masculinise males (Ward, 1972), possibly due to a decrease in testosterone, this was not the case here. Testosterone concentrations were not altered in the plasma nor brain of PNS offspring. This was in agreement to the previous study where there was no change in plasma testosterone observed for PNS offspring (Ashworth et al., 2016), when plasma from trunk blood was used for quantification.

4.4.6 Differences in neuroactive steroid concentrations in plasma and brain regions

Throughout the chapter, there are differences observed in concentrations between circulatory amounts of steroids and those that are in the brain. In previous studies using male rats, steroids such as pregnenolone, DHEA and oestradiol are known to be 6-10 times greater in the hippocampus as compared to those in the plasma (Hojo et al., 2004, Mukai et al., 2006).

Here, it was established that while corticosterone concentrations were always greater in the circulation than in the brain, DOC concentrations were not different between the brain and in the circulation. For allopregnanolone, concentrations were

greater in the brain than the plasma in males, but were greater in female plasma as compared to the female brain. For the other steroids, concentrations were either generally greater in the brain as compared to the plasma, although for some steroids and brain regions, these differences were only significant after acute stress (Appendix C, Table C2). In general, it has been suggested that local synthesis in the brain occurs when steroid levels are greater in the brain than in the circulation (Taves et al., 2010), again attesting to the fact that neuroactive steroids are produced *de novo* in the brain, especially after an acute stressor. Previously, it has also been shown that there is a correlation between plasma and brain steroid precursors, but not for downstream, 3α,5α-reduced steroids (Sze et al., 2018). Therefore, using circulatory steroids as a proxy measurement for brain steroids may not be appropriate, especially for 3α,5α-reduced steroids, which tend to be produced in the brain after an acute stressor.

Regional differences in the brain were also present, where differences between PNS and controls were noted for some brain regions, but not the others (Fig 4.14). Again, given the presence of sub-regions and different cell types within each region, it is difficult to ascribe functional significance to these differences, but it highlights the importance of measuring steroids with regional specificity in mind (i.e. not using whole brain homogenates if possible), and to not to use plasma concentrations to approximate brain levels of neuroactive steroids.

4.4.7 Conclusions and future directions

All in all, these results show that neuroactive steroid concentrations in PNS offspring did not differ to a large extent from the controls, both basally and 30 min following a single 2 min bout of swimming stress, contrary to the hypothesis, although small deficits in the production of 5α- and 3α,5α-reduced steroids were indicated. However, the lack of differences in concentrations of $GABA_A$ positive modulators between controls and PNS may be related to the lack of corticosterone hypersecretion in the PNS offspring this study. It is also worthy to note that the single time-point of sample collection here represents a momentary glimpse at the steroid profile of a rat after an acute stressor, and does not provide information about the timecourse of the stress response. Therefore, in order to obtain more definite conclusions, this study may be repeated using another type of stressor (e.g. restraint stress), and plasma could also be collected via tail nick in order to obtain

samples at multiple time-points, giving us an understanding of how the stress response is resolved. It would also be sensible to carry out the study on female rats where the oestrous cycle stages are taken into account.

Ultimately, this study is only exploratory in nature and measuring steroid concentrations itself is insufficient to determine the role that neuroactive steroids might play. Additional markers can be probed for to confirm and support the results that were observed here, for instance, to establish if a deficit in steroidogenic machinery could have contributed to the compromised responses in PNS animals, quantification of 5α-reductase mRNA expression following swim stress could be carried out in the frontal cortex or adrenal glands.

This study however, contributes and adds weight to the hypothesis that deficits in neurosteroidogenesis might occur in PNS rats. Given these deficits, the possibility of using neuroactive steroids as a therapeutic agent is therefore still feasible, and the LC-MS method used in this study can in fact be used to study effects of such steroid replacement strategies. The absolute concentrations of neuroactive steroids determined in this study can also aid the development of a suitable therapeutic dose to be used in future experiments. Additionally, if a suitable method can be established, it would be of interest to carry out a larger scale metabolomics study profiling a larger number of steroids or even neurotransmitters (Ruoppolo et al., 2018), in order to obtain a fuller picture of how different stress mediators may change relative to each other in PNS offspring as compared to controls.

Chapter 5: Role of steroids in mediating the transmission of stress signals from mother to foetus

5.1 INTRODUCTION

In Chapter 4, the neuroactive steroid profiles in the plasma and brain of control and PNS adult offspring were characterised. Here, the aim was to find out of if these steroids (glucocorticoids and neuroactive steroids) are different between control and stressed groups at foetal stages, which may point to their role in foetal programming. It is hypothesised that there may be (i) foetal overexposure to maternal corticosterone; and (ii) altered levels of neuroactive steroids in the foetal compartment following chronic social stress.

5.1.1 Glucocorticoid metabolism during late pregnancy

Adaptations related to glucocorticoid metabolism during pregnancy have been introduced in Chapter 1. Here, further details with respect to the late pregnancy period where tissues were collected (GD20) will be given.

5.1.1.1 11β-HSD expression pattern in the placenta during late pregnancy

Placental 11β-HSD1 and 11β-HSD2 expression differs in a zone-specific and temporal manner in the placenta (Fig 5.1). Using *in situ* hybridisation, it was previously determined that on GD16, 11β-HSD1 expression is present in the junctional zone (JZ) but barely detectable in the labyrinth zone (LB). Conversely, for 11β-HSD2, on GD16, high expression was observed in the LB, but low expression was observed in the JZ (Waddell et al., 1998, Burton et al., 1996). However, at GD22, the expression of 11β-HSD1 in the LB increased tremendously, whilst for 11β-HSD2, a higher expression was observed in the JZ, but a very low expression in the LB (Fig 5.1) (Burton et al., 1996, Mark et al., 2009, Waddell et al., 1998). When whole placental homogenates were used to measure 11β-HSD2 expression using quantitative PCR, lower 11β-HSD2 mRNA expression was also observed in GD22 rat placentae as compared to those at GD16 (Staud et al., 2006). In the mouse placenta during late pregnancy, a similar pattern is observed in the LB, where 11β-HSD2 expression decreases towards term, concomitant with an increase in 11β-HSD1 (Brown et al., 1996, Thompson et al., 2002). Together, the reciprocal change in placental 11β-HSD1 and 11β-HSD2 expression in late pregnancy is thought to promote the trans-placental passage of corticosterone from the mother to the foetus near term, thereby contributing to the necessary glucocorticoid surge in the foetus before birth (Fowden et al., 1998).

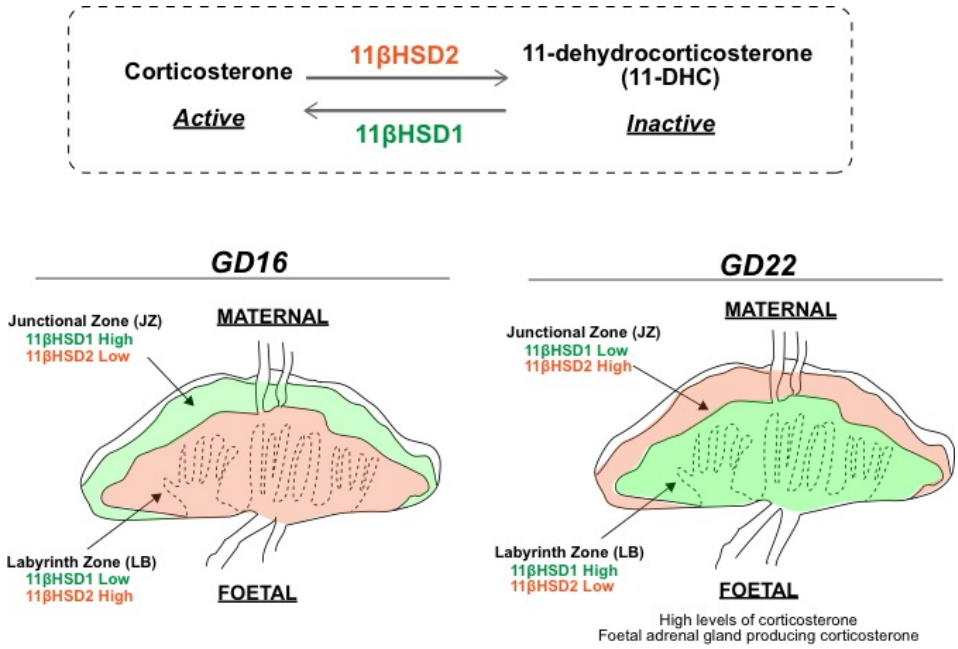


Figure 5.1: Patterns of 11β-HSD1 and 11β-HSD2 expression in the rat placenta during late pregnancy. 11β-HSD1 converts inactive 11-dehydrocorticosterone (11-DHC) to active corticosterone (in the rat), possibly regulating local intracellular glucocorticoid concentrations, while 11β-HSD2 inactivates corticosterone, establishing the "placental glucocorticoid barrier" (Benediktsson et al., 1997, Seckl, 2017). Expression patterns of 11β-HSD1 and 2 in the junctional zone (JZ) and labyrinth zone (LB) are not static and switch between GD16 to GD22. LB contains foetal blood vessels and is the site of maternal-foetal exchange. Data summarised from (Waddell et al., 1998). Decrease in 11β-HSD2 in the LB towards term is thought to promote the glucocorticoid surge.

5.1.1.2 Foetal and maternal plasma corticosterone levels in late pregnancy

Accordingly, foetal rat plasma corticosterone concentrations were also found to rise rapidly from GD17 to 18, peaking at GD19, before falling slightly again before birth (Ward and Weisz, 1984, Cohen et al., 1990, Boudouresque et al., 1988). Apart from the contributory role of the switch in 11β-HSD1 and 11β-HSD2 expression, the foetus' own HPA axis starts to be active around GD18, and corticosterone can be produced by the foetal adrenal glands (Boudouresque et al., 1988).

Maternal corticosterone concentrations are also reported to be the highest during this period (Atkinson and Waddell, 1995). Whilst maternal corticosterone concentrations has always been thought of as exceeding foetal concentrations (Murphy et al., 1974), this is not necessarily the case during late pregnancy. An

investigation into the maternal:foetal corticosterone ratios in late pregnancy in the rat, revealed that foetal baseline plasma corticosterone concentrations in the foetus can be almost six times higher than the maternal values on GD19, but fell to values approximately equivalent to maternal values closer to term (Holt and Oliver, 1968). In another study, Dupouy et al. showed that during GD19 and GD20, the concentrations in the foetuses were respectively 3-fold and 2-fold greater than that of the maternal circulation.

Additionally, it has been proposed that the transfer of corticosterone may not in fact be unidirectional, and there possibly also exists foetal-to-maternal transfer via the umbilical arteries (Boudouresque et al., 1988). This is supported by the finding that adrenalectomising pregnant dams does not drastically affect maternal plasma corticosterone concentrations (Milkovic et al., 1973a), and that maternal corticosterone levels can be correlated to the number of live foetuses *in utero*, suggesting that the foetal adrenal glands make a major contribution to maternal plasma corticosterone concentrations (Dupouy et al., 1975). As such, glucocorticoid metabolism in the foetal, placental and maternal compartments during late pregnancy, and the role of 11β-HSD2 in mediating the intricate balance of glucocorticoid concentrations, appear to be more dynamic and complicated than previously thought, which this study will shed further light on. (Dupouy et al., 1975).

5.1.1.2 MR and GR in the placenta

The placenta is also a target of glucocorticoid action, and is dependent on the expression patterns of its its target receptors MR and GR. MR does not seem to be present in placental tissues, but GR is found in both the JZ and LB of the rat placenta in late pregnancy (Waddell et al., 1998). Whilst *in situ* hybridisation studies show that its expression remains unchanged from GD16 to 22 (Waddell et al., 1998), a later study from the same group using quantitative PCR reported that the expression of GR in the LB increased three-fold from GD16 to 22 (Mark et al., 2009). There is also a substantial increase in placental corticosterone concentrations from GD16 to 22 (Mark et al., 2009), which could imply increased glucocorticoid availability, in addition to increased glucocorticoid sensitivity, in the placenta towards late pregnancy.

Local glucocorticoid action through GR modulates a wide range of functions within the placenta, such as the control of placental vascularity (Hewitt et al., 2006) and its

interaction with steroid hormones. For instance, glucocorticoids can have a stimulating effect on progesterone production, when investigated in minced rat placentae (Matt and Macdonald, 1985), yet at the same time, GR in the rat placenta may also be a target of progesterone action (Ogle et al., 1989). In mice, the loss of GR can alter the methylation pattern of the murine placenta genes profoundly and in a sex-dependent manner (Schmidt et al., 2019a), suggesting that the placenta is not only a regulator of steroid and glucocorticoid metabolism in the foetal component, but itself is also an important target of glucocorticoids.

5.1.1.3 Glucocorticoid metabolism in the foetal liver and foetal brain

An increase in corticosterone concentrations in foetal tissues is required for the foetal growth spurt and organ maturation towards the end of pregnancy. GR is also widely expressed in the developing rat brain (Kitraki et al., 1997), rendering the foetal brain extremely sensitive to changes in glucocorticoid levels. There is a gestational stage specific-pattern of 11β-HSD1 and 11β-HSD2 expression in the developing foetal brain, allowing intricate control of final maturation of neurones (reviewed in (Chapman et al., 2013, Seckl, 1997)).

Brain 11β-HSD2 expression declines from mid-to late-gestation (Diaz et al., 1998). 11β-HSD1 on the other hand, cannot be detected in the foetal brain until GD16, but increases with gestational age, during the time where 11β-HSD2 is declining (Diaz et al., 1998, Moisan et al., 1992), though one report failed to detect 11β-HSD1 mRNA in foetal brain at all (Thompson et al., 2002). The role of foetal 11β-HSD1 in foetal programming has yet to be investigated, however, they may play a role in controlling local concentrations of corticosterone by reactivating 11-DHC that is produced from the inactivation of corticosterone by placental 11β-HSD2 (Wyrwoll et al., 2011).

5.1.2 Changes in glucocorticoid metabolism following gestational stress

The three lines of defence that can protect against glucocorticoid overexposure in the foetus were reviewed in Chapter 1 (Fig 1.8). However, despite these physiological adaptations, chronic gestational stress may still result in negative foetal programming. It has been therefore proposed that chronic gestational stress could compromise these lines of defence), such that foetal exposure to glucocorticoids would exceed what is considered acceptable at this stage

(Barbazanges et al., 1996). Some examples have been given in section 1.5.4, which will be further developed in this section.

Cortisol levels in human studies:

Maternal cortisol levels are known to be correlated with foetal (Gitau et al., 2004) and newborn cortisol levels (Smith et al., 2011). High maternal endogenous cortisol levels during pregnancy (when measured in blood, saliva and urine) are also linked to high amniotic fluid cortisol levels (Sarkar et al., 2007), and are associated with altered length of gestation and low infant birth weight, which are predictors of poor infant outcomes (reviewed in Duthie and Reynolds, 2013). Whilst the link between maternal distress during pregnancy and poor offspring outcomes (e.g. neurodevelopmental disorders) are known, in line with the DOHaD hypothesis (Monk et al., 2012, Bale et al., 2010), inconsistencies arise on whether these outcomes are mediated via altered maternal and foetal HPA axis and increased cortisol levels following maternal distress (Duthie and Reynolds, 2013). A recent systematic review showed that majority of the studies found no significant associations between prenatal maternal cortisol and child outcomes (Zijlmans et al., 2015). This is probably due to the subjectiveness of “maternal distress” in humans (Dipietro, 2012) and also inconsistencies in the measurement of cortisol, as concentrations can vary depending on how and when samples are collected (Dahlerup et al., 2018). This is especially so during late pregnancy, where maternal HPA axis responses to stress are dampened, maternal distress is not always associated with a greater cortisol level (Voegtline et al., 2013, Obel et al., 2005)., Nonetheless, the fact that most studies do not find significant associations suggests that maternal cortisol is not the sole mediator between perceived stress and child outcomes (Zijlmans et al., 2015).

Apart from maternal distress, several studies have also investigated the effects of antenatal synthetic glucocorticoid administration (e.g. dexamethasone or betamethasone) in pregnancies with pre-term birth risk. Synthetic glucocorticoid administration aids foetal lung maturation, however, their administration is also known to be detrimental to infant growth and development (Ilg et al., 2019). Whilst large doses of synthetic glucocorticoids also lead to the inappropriate activation of GR, these synthetic glucocorticoids are not metabolised in the same manner as

cortisol and are not subject to regulation from placental 11β-HSD2, therefore do not directly mimic the action of cortisol in foetal programming.

Placental 11β-HSD2 expression in human studies:

Reduced placental 11β-HSD2 activity is linked to a range of pathological conditions during pregnancy, such as preeclampsia, preterm birth and intrauterine growth restriction (IUGR), presumably due decreased inactivation of maternal cortisol in the placenta and subsequently, increased exposure of the foetus to maternal cortisol (O'Donnell et al., 2009). Offspring of women who consumed large amounts of liquorice, which contains an 11β-HSD2 inhibitor glycyrrhetinic acid, also exhibited impairments in cognitive development and HPA axis function (Raikkonen et al., 2010). There is also evidence that placental 11β-HSD2 is sensitive to maternal distress and most studies report decreased placental 11β-HSD2 expression with maternal distress, possibly as an outcome of the hypermethylation of the *HSD11B2* gene promoter (Monk et al., 2016a). Nonetheless, there are conflicting results as well, as placental 11β-HSD2 expression is found to be upregulated with materal depression (Reynolds et al., 2015a), whilst in another study, maternal anxiety decreases 11β-HSD2 expression, but to a greater extent than maternal depression (O'Donnell et al., 2012). Apart from being sensitive to maternal mood, male and female placenta can be affected differently, and female offspring tend to be more affected than males, showing greater HSD11B2 methylation and also lower mRNA expression as compared to that of males (Green et al., 2015, Mina et al., 2015).

Corticosterone levels in rodent studies:

Studies investigating foetal corticosterone concentrations following stress are more easily carried out in rodents. Circulatory foetal corticosterone concentrations in the rat have been shown to increase following exogenous maternal corticosterone injection (Montano et al., 1993, Bingham et al., 2013), or with stressors that increase maternal corticosterone, such as exercise (Carlberg et al., 1996), tail shock (Takahashi et al., 1998), immobilisation stress (Erisman et al., 1990, Bingham et al., 2013), or immune stress (Cui et al., 2011). Although there are variations in (i) the time elapsed before tissues were collected following the stressor and (ii) the gestational day in which the tissues collected, most studies report a significant increase in foetal corticosterone concentrations, concomitant with the significant increase in maternal corticosterone levels, although a closer investigation into

absolute values reveals that fold changes within maternal groups always tend to be greater (Montano et al., 1993, Bingham et al., 2013, Carlberg et al., 1996, Cui et al., 2011). The mechanisms underlying these changes in foetal corticosterone are not always clear, but changes in placental 11β-HSD2 function are thought to contribute.

Placental 11β-HSD2 expression in rodent studies:

Similar to findings observed in human studies, several rodent studies have reported that chronic stress can decrease the expression and activity of placental 11β-HSD2, which implicates increased transfer of maternal corticosterone to the foetus. Chronic unpredictable stress from GD11 to 20 was found to reduce 11β-HSD2 activity and mRNA expression in the rat placenta (Mairesse et al., 2007), whilst maternal undernutrition during late gestation reduces 11β-HSD2 mRNA expression at term (Lesage et al., 2001). Likewise, it has been suggested the decrease in placental 11β-HSD2 expression occurs through epigenetic mechanisms, as increased methylation was found in several sites of the *Hsd11b2* gene promoter following chronic restraint stress in pregnant rats from GD14 to 20 (Jensen Peña et al., 2012). However, there are also reports which contend that 11β-HSD2 expression increases in response to stress. Using a mouse model, it was found that an increase in maternal corticosterone released from an osmotic pump can alter normal placental growth and development in a sex and gestational stage-specific manner (Cuffe et al., 2012). Whilst glucocorticoid receptor expression mRNA expression was increased at both E14.5 and E17.5 following maternal corticosterone overexposure, 11β-HSD2 mRNA expression was increased in E14.5 but decreased instead in E17.5 (Cuffe et al., 2012). In another model where pregnant mice were treated with betamethasone, a synthetic glucocorticoid, an increase in 11β-HSD2 protein expression was observed in all stages of pregnancy investigated from E11.5 to E17.5 (Ni et al., 2018).

The duration and nature of the stress seems to affect how 11β-HSD2 expression changes as well, as it was found that whilst acute restraint stress increased rat placental 11β-HSD2 activity, a chronic stress regime prior to the acute stressor abolished this increase (Welberg et al., 2005). It was thus proposed that the upregulation of 11β-HSD2 could be an adaptive response to a short term elevation of maternal glucocorticoids, while chronic exposure may lead to reduced placental 11β-HSD2 instead. Sex-dependent changes were also noted, for instance, where

prenatal alcohol exposure for two weeks resulted in a decrease in 11β-HSD2 mRNA levels in female rat placenta, but increased 11β-HSD2 mRNA in male placentae on GD21 (Wilcoxon et al., 2003).

Prenatal social stress model:

Previous studies using the prenatal social stress model have established that despite attenuated HPA axis reactivity in late pregnancy, corticosterone secretion is greater in stressed rat dams compared to non-stressed dams (Brunton and Russell, 2010). However, it has not been investigated in this model, whether maternal social stress results in increased corticosterone in the foetal circulation or altered placental 11β-HSD2 expression. In fact, in many of the earlier experiments reviewed above, quantification of 11β-HSD2 expression (either gene or protein expression) was typically never carried out in tandem with foetal corticosterone measurements. For instance, even in 11β-HSD2 global knockout mice, where placental 11β-HSD2 is not expressed, it has not been established whether foetal circulatory corticosterone concentrations are affected or whether there is increased maternal-foetal corticosterone transfer (Wyrwoll et al., 2009, Holmes et al., 2006). The concentrations of the inactive metabolite 11-DHC were also rarely quantified in previously published experiments, therefore conclusions about the role of placental 11β-HSD enzyme in glucocorticoid conversion cannot be drawn. Therefore, this study aims to elucidate whether both foetal corticosterone and 11-DHC concentrations are affected following maternal social stress, and if the observed patterns could be linked to changes in 11β-HSD2 expression patterns in the placenta.

Placental GR:

The actions of glucocorticoids are mediated not only by their circulating levels, but also by their local metabolism (via 11β-HSD enzymes) and intracellular signalling through MR and GR. Changes in placental GR gene expression have been associated with compromised pregnancies in humans, where for instance, a decrease in placental GR gene expression is associated with maternal depressive symptoms (Reynolds et al., 2015b). In rodents, patterns of GR protein expression following gestational stress seem to be variable and are dependent on the characteristics of the dam. In socially dominant mice dams, restraint stress for three days elevated placental GR and 11β-HSD2 protein expression, whilst in socially

submissive mice, GR protein expression was reduced without changes in 11β-HSD2 protein expression (Gross et al., 2018). Placental GR mRNA expression was therefore also quantified in this study using *in situ* hybridisation, as changes could imply altered glucocorticoid sensitivity of the placenta, which can affect the outcomes of the pregnancy.

5.1.3 Changes in neuroactive steroid concentrations in placental and foetus following gestational stress

Neuroactive steroids in the foetal compartment regulate growth and development of the central nervous system, ensuring the right amount of inhibitory and excitatory signals at the right time, for proper neurogenesis, neuronal survival, myelination and neurotoxicity (section 1.4.3). Changes in their levels in the brain, or levels in the placenta (which can be a source, or possibly provides the precursors; section 1.4.2) following stress could result in detrimental effects in the offspring.

Human studies:

In humans, one study showed that the 5α-reductase (both isoforms 1 and 2) protein expression was lower in placental tissues from pre-term pregnancies, compared with term pregnancies (Vu et al., 2009). Yet at the same time, placental 5α-reductase expression was positively correlated to foetal cortisol levels, suggesting a potential link between foetal HPA axis function and placenta steroidogenesis (Vu et al., 2009).

Rat studies:

In GD19 male rat foetuses, reduced 5α-reductase type 1 activity is observed in the cerebral cortex and hypothalamus when their mothers are stressed by immobilisation on GD15 to 18 (Ordyan and Pivina, 2005). Another form of stress, uteroplacental insufficiency, induced by bilateral uterine vessel ligation, results in rat foetuses having increased brain allopregnanolone concentrations compared with control foetuses on GD20 (Westcott et al., 2008). However, at postnatal day 6, growth restricted rat offspring show reduced brain allopregnanolone concentrations compared to controls, which indicate there may be some loss of steroidogenic capacity in the neonatal adrenal gland during gestation, but deficits only surface during the postnatal period due to the loss of precursors from the placenta (Westcott et al., 2008).

Other animal species:

The majority of studies investigating foetal steroidogenesis in the brain have been carried out in species with longer gestation periods than the rat (22 days), such as the sheep (150 days) and guinea pig (70 days). The levels of allopregnanolone in the foetal sheep brain are extremely high, exceeding those found in the postnatal and adult brain, whilst steroidogenic enzymes are also present and increase steadily towards term (Nguyen et al., 2003b). The high allopregnanolone content protects the foetal sheep brain from traumatic insults and excitotoxic injury (Yawno et al., 2007), but also suppresses CNS activity, maintaining the low level of arousal-like behaviour in foetuses (Nicol et al., 1997, Crossley et al., 1997).

Allopregnanolone concentrations in the offspring are also affected by prenatal stress, and the direction of change is likely to be dependent on the stressor (Hirst et al., 2006). Acute asphyxia induced by 10 min of umbilical cord occlusion results in increases in allopregnanolone concentrations in both brain tissue and brain extracellular fluid of foetal sheep, accompanied by increased 5α-reductase type 2 and p450scc protein expression in the brain (Nguyen et al., 2004). However, no increase in allopregnanolone concentrations are observed when the stressor was longer in duration, for instance, where chronic hypoxia is induced using microsphere embolisation of the sheep placental arteries for a period that ranges from 17 to 23 days (Nguyen et al., 2003a).

In the foetal guinea pig brain, high levels of allopregnanolone are also observed towards term (Kelleher et al., 2011). Repeated betamethasone administration during the guinea pig gestation results in reduced 5α-reductase 2 expression in the foetal hippocampus and in the placenta (McKendry et al., 2010). Inhibiting neuroactive steroid production during gestation using the 5α-reductase inhibitor, finasteride, also results in increased anxiety-like behaviour in the female offspring during adulthood, implying its importance in foetal brain development (Cumberland et al., 2017). Whilst maternal allopregnanolone treatment increases foetal plasma allopregnanolone levels, prenatal stress prevents this increase, suggesting that neuroactive steroid production, transfer or metabolism could be disrupted following prenatal stress (Bennett et al., 2013).

Prenatal social stress model:

It is not known whether similar effects occur in the rat maternal social stress model. In Chapter 4, it was demonstrated that the PNS offspring did not display altered neuroactive steroid concentrations in the brain in adulthood under basal conditions, and only slight deficits were observed following stress exposure. Nonetheless, altered neuroactive steroid production may have occurred during foetal development, impairing proper brain development, indirectly leading to the development of other aberrant phenotypes (summarised in Fig 1.7) such as anxiety and HPA axis dysregulation in adulthood.

5.1.4 Role of sex steroids during foetal development

Sex steroids in foetal development are involved not only in sexual differentiation (section 1.3.4), but also control neuronal growth and synaptogenesis, dendritic branching and myelination (Garcia-Segura and Melcangi, 2006, Haraguchi et al., 2012). Sex hormone alterations during foetal life are known to induce permanent alterations in the brain architecture (Nugent et al., 2011). Given the sex differences observed in the PNS offspring, where adverse programming seems to affect males to a greater extent than females (Brunton and Russell, 2010), sex hormone changes could underlie these sex-specific outcomes of prenatal stress.

Progestogens:

During foetal development, progesterone plays a role in ensuring normal neuronal development in both male and female foetuses, by promoting myelination for instance (Garcia-Segura and Melcangi, 2006). Although there does not appear to be sex differences in foetal plasma progesterone concentrations in the rat (Weisz and Ward, 1980), progesterone receptor distribution and expression are reported to be very different in males and females, suggesting that progesterone action could play an important role in sexual differentiation of the brain (reviewed in (Wagner, 2008)).

Androgens:

A testosterone surge occurs in male rat foetuses at GD18-19, and sex differences in plasma testosterone are also observed to be greatest at this stage (Weisz and Ward, 1980, Habert and Picon, 1984), representing a critical point in the sexual differentiation of the male foetal brain (McEwen, 1981, Perakis and Stylianopoulou, 1986). Prenatal stress has been suggested to alter the characteristics of the

testosterone surge (Ward and Weisz, 1980, Barrett and Swan, 2015), either resulting in a premature surge on GD17 instead, or lower peak concentrations of testosterone (Ward et al., 2003). Previous rat studies investigating the impact of gestational stress on testosterone levels showed that two days of maternal lipopolysaccharide (LPS) endotoxin administration decreases testosterone in male foetuses on GD19 (Cui et al., 2011), whilst chronic immobilisation stress increases testosterone levels only in male foetuses on GD17, but not during the testosterone surge on GD18-19 (Ward and Weisz, 1984).

The placenta also contains androgen receptors (AR) and AR-mediated testosterone action on the placenta could lead to deleterious outcomes such as preeclampsia (Kumar et al., 2018). In humans, foetal testosterone concentrations correlate with both foetal and maternal cortisol concentrations, thus stress and the associated changes in cortisol secretion, may interact with androgen actions and contribute to sex-specific programming effects (Gitau et al., 2005).

5.1.5 Aims of this chapter

Gestational social stress using the modified resident-intruder paradigm is administered to pregnant rats from GD16 to GD20 during late pregnancy, when the changes in glucocorticoid metabolism are occurring (as explained in section 5.1.1). Whilst past studies have investigated the effects of this chronic social stress the offspring in adulthood, the effects of the form of stress on the placenta or foetus during pregnancy have yet to be investigated. In this study, pregnant rats were killed at GD20 and tissues from the pregnant dam, placental and foetus were collected. Various aspects of glucocorticoid metabolism were first investigated via LC-MS quantification of corticosterone and 11-DHC, as well as *in situ* hybridisation for genes involved in glucocorticoid metabolism (11β-HSD1 and 2) and action (GR). Additionally, a full neuroactive steroid profile, similar to that carried out in the adults was determined in the placenta and foetal tissues. A summary of the changes occurring during late pregnancy is presented in Figure 5.2, as well as the specific questions for this study.The specific aims of this chapter are to determine if there are differences between stressed and non-stressed groups at GD20, with respect to:

1. Maternal and foetal circulatory corticosterone and 11-DHC concentrations
2. 11β-HSD2 mRNA and protein expression in the placenta
3. GR mRNA expression in the placenta
4. Corticosterone and 11-DHC concentrations in the foetal liver and foetal brain
5. Local glucocorticoid metabolism in the foetal hippocampus (by quantifying 11β-HSD1 and 11β-HSD2 mRNA expression)
6. The steroidal profile in the placenta, foetal liver and foetal brain

It is hypothesised that following chronic stress, maternal corticosterone concentrations increase, whilst a concomitant decrease in 11β-HSD2 expression occurs in the placenta. These two events together result in increased foetal overexposure to corticosterone, where greater corticosterone concentrations will be observed in plasma, liver and the brain of stressed foetuses as compared to controls. Local glucocorticoid regulation in the developing foetal brain (11β-HSD1 and 2 expression) may also be altered. Secondly, it is also hypothesised that there may be a decrease in neuroactive steroid concentrations in the developing foetal brain, indicating compromised neuroactive steroid-mediated protection.

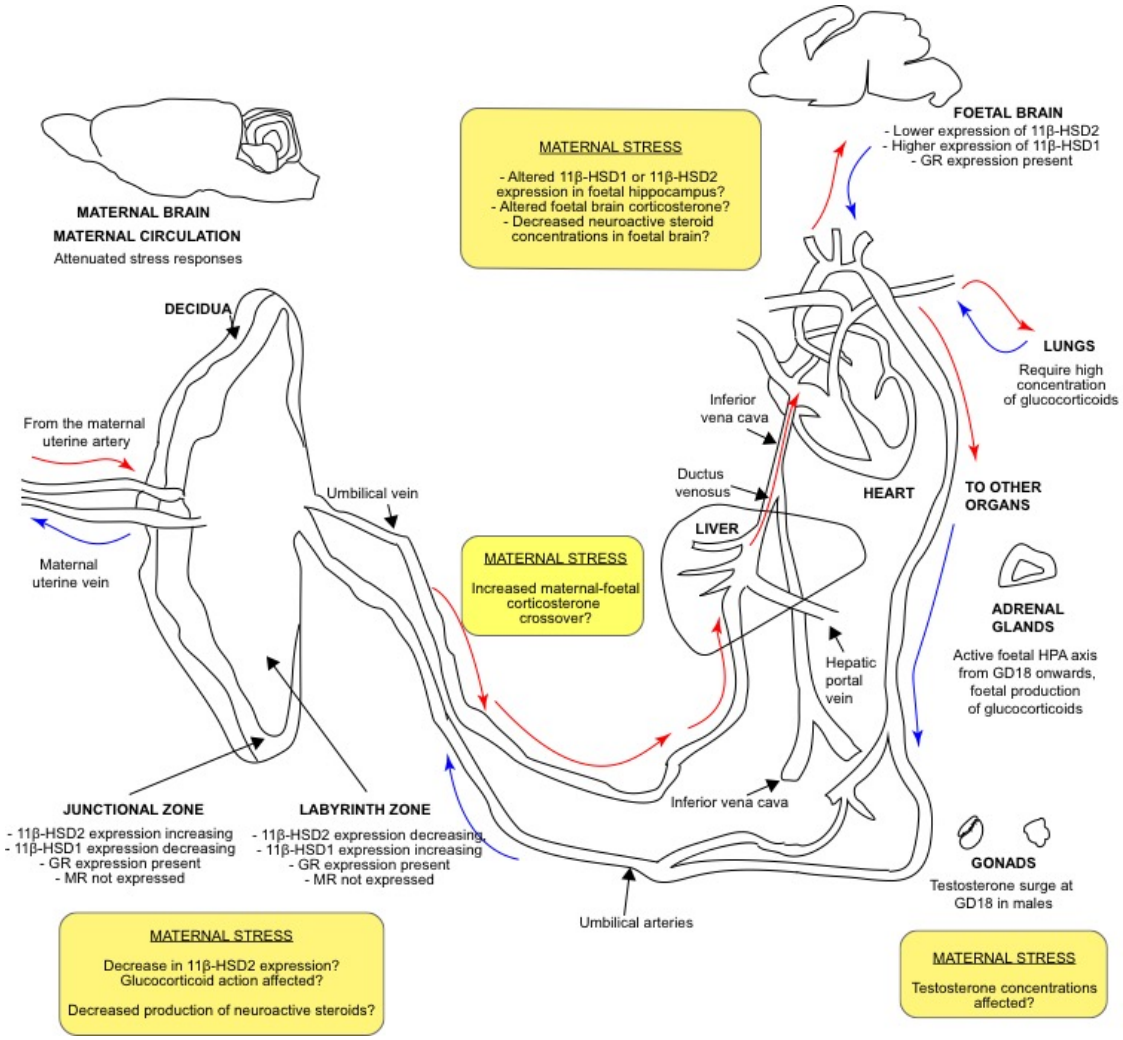


Figure 5.2: Summary of physiological changes in the pregnant rat dam, placenta and foetus occurring at GD20 and potential changes to be investigated following gestational stress. To recap, the placenta is the site of maternal-foetal exchange, and contains both maternal and foetal blood vessels in the labyrinth zone. Blood leaving the placenta on the foetal side enters the umbilical vein and enters the foetal liver directly, and a portion of the blood is supplied to the rest of the body via the ductus venosus. Adaptations that are characteristic of late pregnancy are listed below each organ, whilst potential changes following gestational stress which are investigated in this chapter are listed in the boxes in yellow. Oxygenated blood flow is represented in red, while deoxygenated blood flow is represented in blue.

5.2 METHODS

5.2.1 Animals, social stress and tissue collection

The generation of pregnant rats and social stress were carried out as described in Chapter 2. Dams in the stressed group (7 dams) underwent social stress using the modified resident-intruder-paradigm from GD16 to 20 of gestation, and were killed immediately after the last stressor on GD20 (approximately 10 min after the induction of stress). Unstressed control dams (7 dams) were removed from the home cage immediately and killed. Social stress and tissue collection was carried out between 10:00 – 14:00 each day.

Following decapitation, tissue collection occurred swifly, detailed in Chapter 2 (section 2.3), with the assistance of Dr Paula Brunton. Brieftly, trunk blood was collected from pregnant dams following conscious decapitation, into chilled collection tubes containing 0.5% (w/v) EDTA kept on ice. The foetuses and placenta were quickly removed from the dam and the sex was determined (Chapter 2). Foetuses were decapitated and trunk blood was collected using EDTA-coated capillary collection tubes, also kept on ice. Foetal brains, maternal and foetal liver were then collected. Placenta, foetal brains and liver were frozen on dry ice, and stored at -80^{o}C until further use. Both maternal and foetal trunk blood were centrifuged at 1500 g for 20 min at 4^{o}C, and the plasma was separated and stored at -20^{o}C until further use.

The experimental plan outlined above, including the various biochemical markers investigated, is summarised in Figure 5.3.

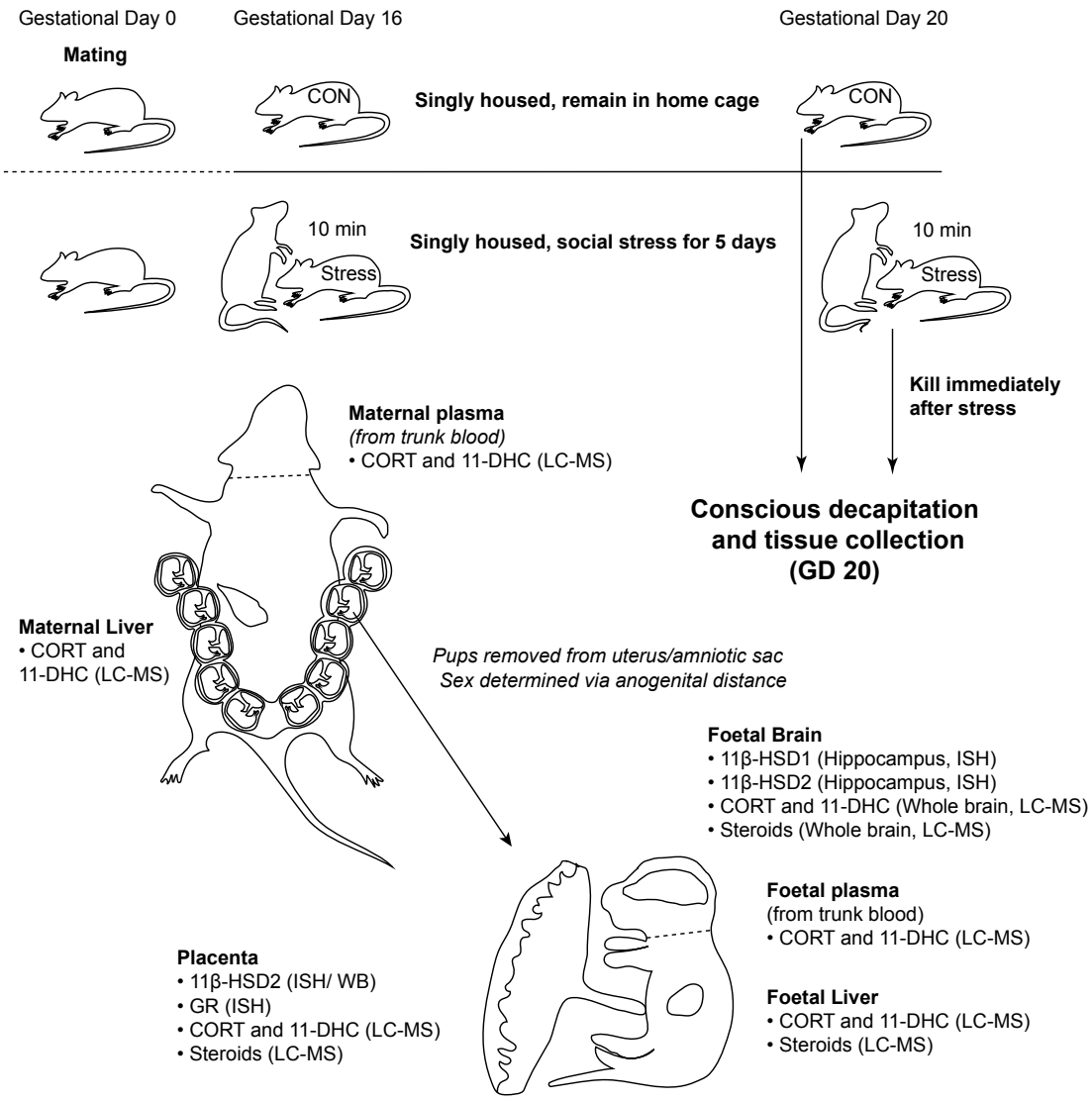


Figure 5.3 Experimental plan for this study. Pregnant dams were culled on gestational day (GD) 20, following which placental, maternal, and foetal tissues and plasma were collected. The biochemical tests carried out using each tissue is listed below each tissue type. CON: Control, CORT: Corticosterone, 11-DHC: 11-dehydrocorticosterone, GR: Glucocorticoid receptor, ISH: In situ hybridisation, WB: Western blotting

5.2.2 LC-MS quantification of steroids

LC-MS quantification of steroids was carried out in as described in Chapter 2 (section 2.5) and 3. Maternal and foetal plasma used for the quantification of corticosterone and 11-DHC were processed separately from the other tissues, and were subjected to a modified LC-MS detection method. Maternal liver, placenta, foetal liver and foetal brain were processed as described in Chapter 3 for steroid quantification. The steroids quantified were corticosterone, 11-DHC, DOC, DHDOC, THDOC, progesterone, DHP, allopregnanolone, pregnenolone and testosterone.

Sample processing for maternal and foetal plasma: Both maternal plasma and foetal plasma were first diluted 1:100. 100 µL of 1:100 diluted plasma was used for LC-MS quantification of steroids. 7 calibration standards for corticosterone and 11-DHC were prepared as previously described with serial dilution, however, the surrogate matrix used was 0.2% BSA to mimic the albumin concentrations in the 1:100 diluted plasma. The samples were processed as described in section 2.5, where 100 µL of diluted plasma were used, extracted twice with methanol/1% formic acid, and underwent C18 solid phase extraction. All maternal (n=7, two groups) and foetal plasma samples (n=6-7, four groups) were processed on the same day using the same standard curves. All samples were derivatised within a single batch using 1 mg/ml Girard's T reagent, in methanol/ 0.2% formic acid, dried and reconstituted in 50% methanol.

LC-MS method for corticosterone and 11-DHC only: A modified gradient was used for the detection of corticosterone and 11-DHC only, using diluted maternal and foetal plasma, with a shorter run time of 10 min (see Figure 3B). Multiple reaction monitoring was carried out, but only corticosterone (460.2→401.1), 11-DHC (458.2→399.2) and corticosterone-d5 (464.3→405.1) were monitored. Area under the curves for corticosterone and 11-DHC were normalised to corticosterone-d5 (internal standard), and the ratios were used to construct the calibration curves and extrapolate corticosterone and 11-DHC concentrations from samples.

Sample processing for maternal liver, placenta, foetal liver, and foetal brain: A small sample of maternal and foetal liver tissue (approx. 50 mg) was excised and used for sample processing. 1/8th of a placenta (containing both JZ and LB) and one hemisphere of a foetal brain (containing both cortex and the brainstem) were used.

Samples were cut and weighed while frozen, on dry ice. Sample processing was carried out as described in section 2.5.

LC-MS method for steroids panel: A full gradient was used for the detection of the steroids panel, which also includes corticosterone and 11-DHC (see Figure 3A). Transitions were monitored as detailed in Table 3.4.

5.2.3 ACTH quantification in maternal plasma

Total ACTH in the maternal plasma were measured using the MP Biomedicals ACTH Double Antibody RIA Kit (Cat. 07106102) as per the manufacturer's instructions, by Dr Paula J Brunton. 100 µL of undiluted plasma sample kept in -20^{o}C was used, and samples were run in duplicate.

5.2.4 *In situ* hybridisation

In situ hybridisation was carried out to probe for placental 11β-HSD2 and GR mRNA expression, and foetal hippocampal 11β-HSD1 and 11β-HSD2 mRNA expression. Probes were kindly donated by Prof Megan Holmes and Prof Karen Chapman (Table 5.1).

Tissue sectioning: Tissue sectioning was carried out by MSc student Joana Fernandes. Frozen tissue was mounted (but not embedded) on OCT (Tissue-Tek) and 16µm sections were cut using a cryostat at -19^{o}C (Leica CM1850), then thaw-mounted on Polysine adhesion slides (ThermoScientific). Foetal brains were cut at the level of the hippocampus, while placentae were cut transversely, ensuring both JZ and LB were represented in each section. Every 4th section was collected on each slide, and each slide contained 8 sections. Marker sections were collected on gelatine subbed slides, and fixed with acetic alcohol fixative (4% w/v formaldehyde and 5% v/v acetic acid in ethanol) and stained with 1% toluidine blue for visualisation. Tissue sections on slides were stored at -80^{o}C until processing for *in situ* hybridisation.

In situ hybridisation: In situ hybridisation was carried out as described in Chapter 2, with specific conditions for each probe detailed in Table 5.1.

Target mRNA	Source	Restriction enzymes	Plasmid vector; Size of insert	RNA Polymerase	Exposure time
GR	(Seckl et al., 1990)	AS: AvaI S: EcoRI	620 bp	AS: T7 S:SP6	4 weeks
11β-HSD1	(Agarwal et al., 1989)	AS: NotI S: KpnI	616 bp	AS: T7 S: SP6	5 weeks
11β - HSD2	(Leckie et al., 1995)	AS: SphI S: SalI	750 bp	AS: SP6 S: T7	4 weeks

Table 5.1: Conditions for *in situ* hybridisation. AS: Antisense, S: Sense. Hybridisation was carried out at 55°C for all three probes, while post-hybridisation washes consisted of the first step of 2x SSC at RT for 30min, followed by 0.1x SSC at 60°C for 50 min three times.

Sense probes, which do not bind to the mRNA sequence, were used as negative controls and processed as described above. For 11β-HSD1 and 11β-HSD2, sense probes were applied to test kidney and placental tissue sections to determine the specificity of binding. Sections hybridised with sense probes showed no signal above background (Figure 5.4).

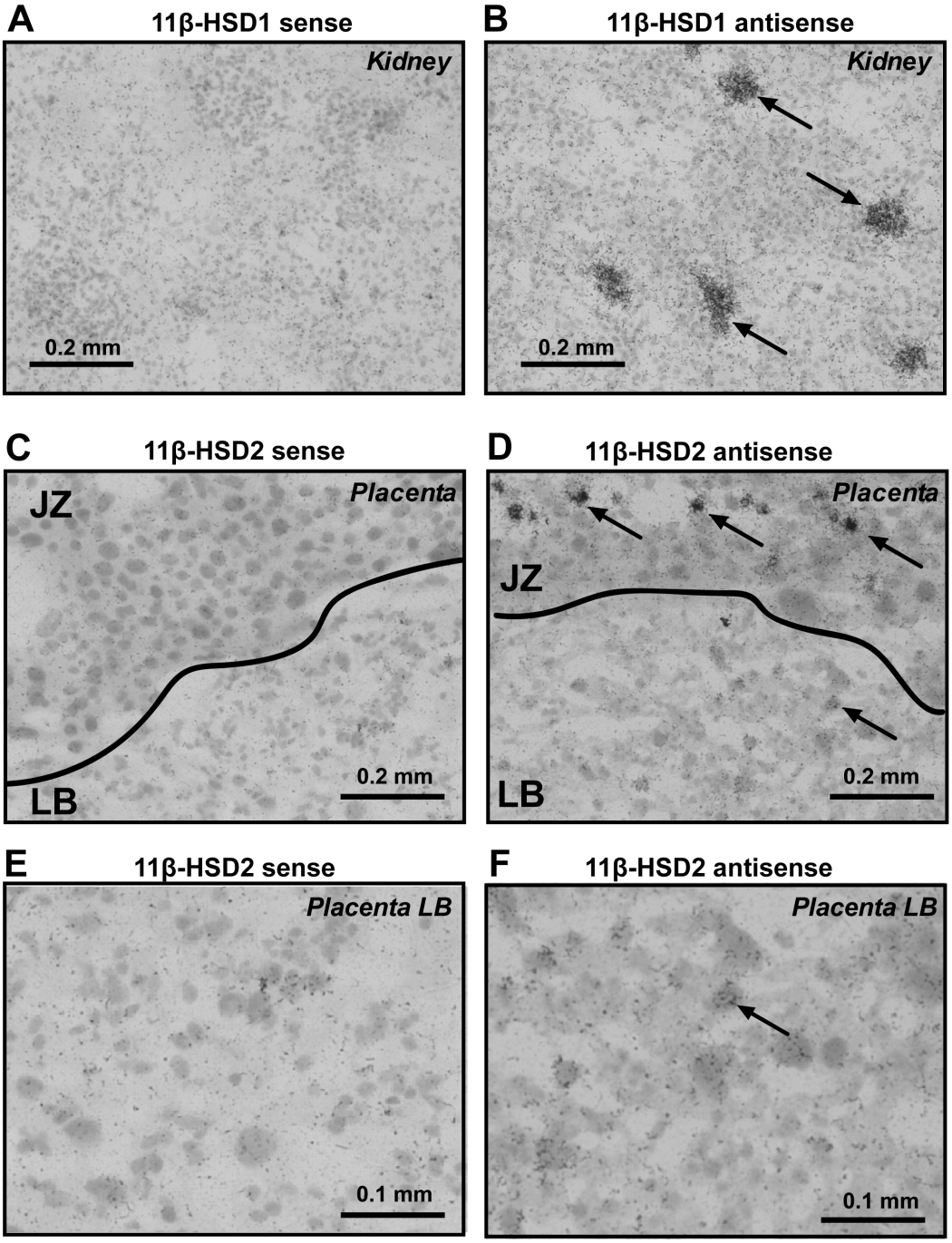


Figure 5.4: Representative figure of the comparison between antisense and sense probes. Clusters where silver grains were super imposed on top of cells (black arrows) represent cells expressing the target mRNA. Positive cells were observed for antisense probes for 11β-HSD1 (B) and 11β-HSD2 (D and F). No signal was detected for respective sense probes (A, C and E). As the expression of 11β-HSD2 in the labyrinth zone (LB) is lower and fainter than that of the junctional zone (JZ), E and F show these cells at a higher magnification. Positive cells in (F) (black arrow) have silver grains at a density 5x higher than that of the background and than that of the sense probes (E) where the black clusters were not specific, and not clustered on top of cells. Photomicrographs are taken at 10x magnification.

Data analysis and quantification: Placental 11β-HSD2 (both JZ and LB) was quantified by Ms Joana Fernandes. Images were taken at 20X magnification, from one section per placenta. For the JZ, data was analysed from 13-18 images cross the entire JZ. Grain density was measured by dividing the sum of the area containing silver grains over the sum of the total area of the JZ for each image. For the LB, 4 images were taken across the LB and the grain density was measured by dividing the area containing silver grains over the total area sampled, and were averaged across the four images. 11β-HSD1 and 2 in the foetal hippocampus were quantified with images taken at 20X magnification. For each foetal brain, 7-8 sections were analysed, where one image was taken per section at the Ammon's horn region, which approximately translates to the CA2 region of the GD20 hippocampus. Grain density was measured using Fiji software, with the method detailed in section 2.7.5 and were averaged across the 7-8 sections.

5.2.5 Western blotting

Western blotting was carried out for the detection of 11β-HSD2 protein expression in the placenta.

Sample homogenisation and protein quantification: $1/8^{th}$ of a placenta, comprising both JZ and LB, was excised and homogenised on ice in 1.5 mL Eppendorf tubes using a handheld homogeniser in RIPA lysis buffer (Pierce, ThermoFisher) with a protease inhibitor (HALT inhibitor cocktail, ThermoFisher). The homogenate was centrifuged at 4^{o}C for 20 min at 10000 g, and the supernatants were removed and their protein concentration quantified using a bicinchroninic (BCA) assay (Micro BCA Protein Assay Kit #23235, ThermoFisher). Standard calibrants with concentrations of 125 – 2000 µg/ml were serially diluted from 2 mg/ml BSA protein standards (Sigma). The working solution was prepared by mixing reagent A, B and C in the ratio A:B:C=25:24:1 respectively, and mixed with the sample in a 1:20 sample:reagent ratio. Samples were diluted 1:40 with PBS and 10 µL was aliquoted into 96-well plates in triplicate. 200 µL of working solution was added and the plate was placed on a shaking platform for 1 min, then incubated at room temperature for 2 hr. Optical density was read at 570 nm, on a Synergy HT spectrophotometer. A linear standard curve (y=mx + c) was generated in Excel and the protein concentration of each sample was determined.

SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis): To ensure equal sample loading, samples were diluted to a loading concentration of 50 µg with RIPA Lysis buffer. NuPAGE reducing agent (10x) and loading buffer (4x; LDS sample buffer containing loading dye) were then added in appropriate ratios to each sample and incubated at 70°C for 10 min. Samples, together with a MagicMark XP protein ladder, were loaded in pre-cast NuPAGE 4-12% Bis-Tris protein gels, secured on the XCell SureLock Mini Cell platform. 1X NuPAGE MOPS SDS Running Buffer was used, and the run voltage was set at 85V for 15 min and then at 185V until the dye front reached the bottom of the gel. All reagents were from ThermoFisher Scientific.

Semi-dry transfer: Following SDS-PAGE, gels were equilibrated in transfer buffer for 10 min (0.1M Tris, 0.2M glycine, 5% (v/v) methanol). Meanwhile, Immobilon-FL PVDF Membrane (Merck Millipore, Livingston, UK) was reactivated in methanol, and also placed in transfer buffer for 10 min for equilibration. Semi-dry transfer was performed in a Novex Semi-Dry Blotter (ThermoFisher Scientific), where the gel and membrane was sandwiched between filter paper stacks soaked with transfer buffer. Transfer was carried out at 280 mA for 1 hr. Membranes were air-dried after transfer to permanently bind proteins to the membrane.

Immunoprobing and detection: PVDF membranes were reactivated in methanol for 2 min, rinsed with ddH_2O, and in PBS for 2 min. Membranes were then blocked in Odyssey blocking buffer (Li-Cor Bioscience, Cambridge, UK) for 1 hr. Primary antibody targeting the 11β-HSD2 protein (Abcam, #ab80317) was diluted in blocking buffer with 0.1% (v/v) Tween (1:250), then incubated with the membrane overnight at 4°C with gentle agitation. The optimal dilution of primary antibody was determined in a trial run. Following overnight incubation, membranes were washed in PBS (10 min x 5 times) and incubated with a fluorescent secondary antibody (Goat anti-rabbit IgG IRDye 680RD, Li-Cor, 1:5000), 1 hr in RT, also diluted in blocking buffer with 0.1% (v/v) Tween-20, protected from light. Following another set of 5 x 10 min washes with PBS, blots were then visualised on the Li-Cor Odyssey Infrared Imaging System, using the ImageStudio software. To probe for the internal loading control β-actin, the blots were then stripped for 20 min using a mild stripping buffer (15 g/L glycine, 1 g/L SDS, 1% v/v Tween 20, protocol from Abcam), then washed twice with PBS. Primary antibody targeting β-actin (Sigma, #A5411), was diluted in blocking buffer with 0.1% (v/v) Tween-20 (1:50000), then added to the membrane

for 1 hr incubation in RT. Membranes were then washed five times with PBS, and then incubated with fluorescent secondary antibody (Donkey anti-mouse IgG IRDye 680 RD, Li-Cor, 1:10000) for another hour in RT. Washes and imaging was carried out in the same way as before, with exposure time modified according to the signal of each of the proteins probed.

Data analysis: Densitometric analyses were carried out on ImageJ (NIH, Washington, DC), using commands from the "Analyze→Gels" submenu, where both the size and the grey density of each band was taken into account. The grey value obtained for the protein of interest was then normalised to the grey value obtained for the loading control. Two technical duplicates were run for every sample (i.e. two independent Western blot runs), and a mean value was taken from both runs.

5.2.6 Statistical analysis

Comparisons between stressed and non-stressed dams were carried out using a Student's t-test, carried out using Prism 6.0 (section 2.6). For the placentae and foetal tissues, two-way ANOVAs were carried out with R-Studio (section 2.6, Appendix A), with stress and sex as the main factors investigated. Pairwise comparisons were carried out using Student's-Newman-Keuls test to determine significant differences between groups. All data are presented as bar graphs with standard error of mean (s.e.m.), overlaid with individual data points to reveal variability. Significance level was set at 0.05 in all cases.

5.3 RESULTS

5.3.1 Glucocorticoids are increased in the maternal circulation but not in the foetal brain following maternal stress

Maternal plasma: Stressed dams had significantly greater plasma concentrations of ACTH (1.4-fold; p=0.0028, t=3.75, df=12; Fig 5.5A), corticosterone (3.7-fold; p=0.0052, t=4.18, df=6.30; Fig 5.5B) and 11-DHC (1.7-fold; p=0.0174, t=3.10, df=6.98, Fig 5.5C) compared to the non-stressed controls.

Maternal liver: Corticosterone and 11-DHC concentrations in the maternal liver of stressed dams were not statisticaly different from that of the control dams (Fig 5.5D-E; p=0.079, t=1.92, df=12 for corticosterone; p=0.23, t=1.30, df=7.93, for 11-DHC).

Placenta: There was a significant main effect of stress on both placental corticosterone concentrations ($F_{1,24}$=7.49, p=0.011) and 11-DHC concentrations ($F_{1,24}$=5.71, p=0.025) but no main effect of sex, nor any stress x sex interactions (Fig 5.5F-G). Post-hoc pairwise comparisons revealed that stressed male placentae had significantly greater concentrations of both corticosterone (p=0.0098) and 11-DHC (p=0.03) as compared to the control male placentae, but this difference was not observed when comparing control and stressed female placentae.

Foetal plasma: There was a significant main effect of stress on foetal plasma corticosterone concentrations ($F_{1,23}$ = 6.89, p=0.015; Fig 5.5H). There was no main effect of sex nor any stress x sex interactions. Post-hoc SNK pairwise comparisons showed that plasma corticosterone concentrations were significantly greater in the stressed female foetuses as compared to the control females (p=0.017), albeit to a smaller extent (1.3-fold) as compared to that observed between the stressed and controls dams (3.7-fold)(Fig 5.5H). In contrast, there was no significant difference in plasma corticosterone between the stressed males and the control males (p=0.259). Plasma 11-DHC concentrations did not differ between control and stressed foetuses for either sex. Absolute concentrations of foetal plasma corticosterone are also three-fold greater than that of the maternal concentrations, while foetal 11-DHC concentrations are two-fold greater than that in the maternal circulation.

Foetal liver: There was a significant main effect of stress on foetal liver corticosterone concentrations ($F_{1,24}$=11.5, p=0.0024; Fig 5.5J), without a main effect of sex nor any interactions. Liver corticosterone concentrations were significantly greater in stressed foetuses compared to control foetuses, for both males (p=0.012) and females (p=0.048). 11-DHC concentrations were not different in any of the four groups (Fig 5.5K). However, there was a significant main effect of stress on 11-DHC:corticosterone ratios in the liver ($F_{1,24}$=5.01, p=0.035, graphs not shown; Male/Control: 0.144 ± 0.040, Male/PNS: 0.070 ± 0.005, Female/Control: 0.143 ± 0.046, Female/PNS: 0.077 ± 0.013), however, post-hoc analyses were not significant (p=0.11 for males, p=0.15 for females). Absolute concentrations of foetal liver corticosterone are also on average six-fold greater than that of the maternal concentrations, while foetal liver 11-DHC concentrations are more than 10-fold higher than that in the maternal liver.

Foetal brain: There were no significant differences in concentrations of either corticosterone or 11-DHC in the foetal brain (Fig 5.5L-M). 11-DHC:corticosterone ratios were not different between all four groups as well.

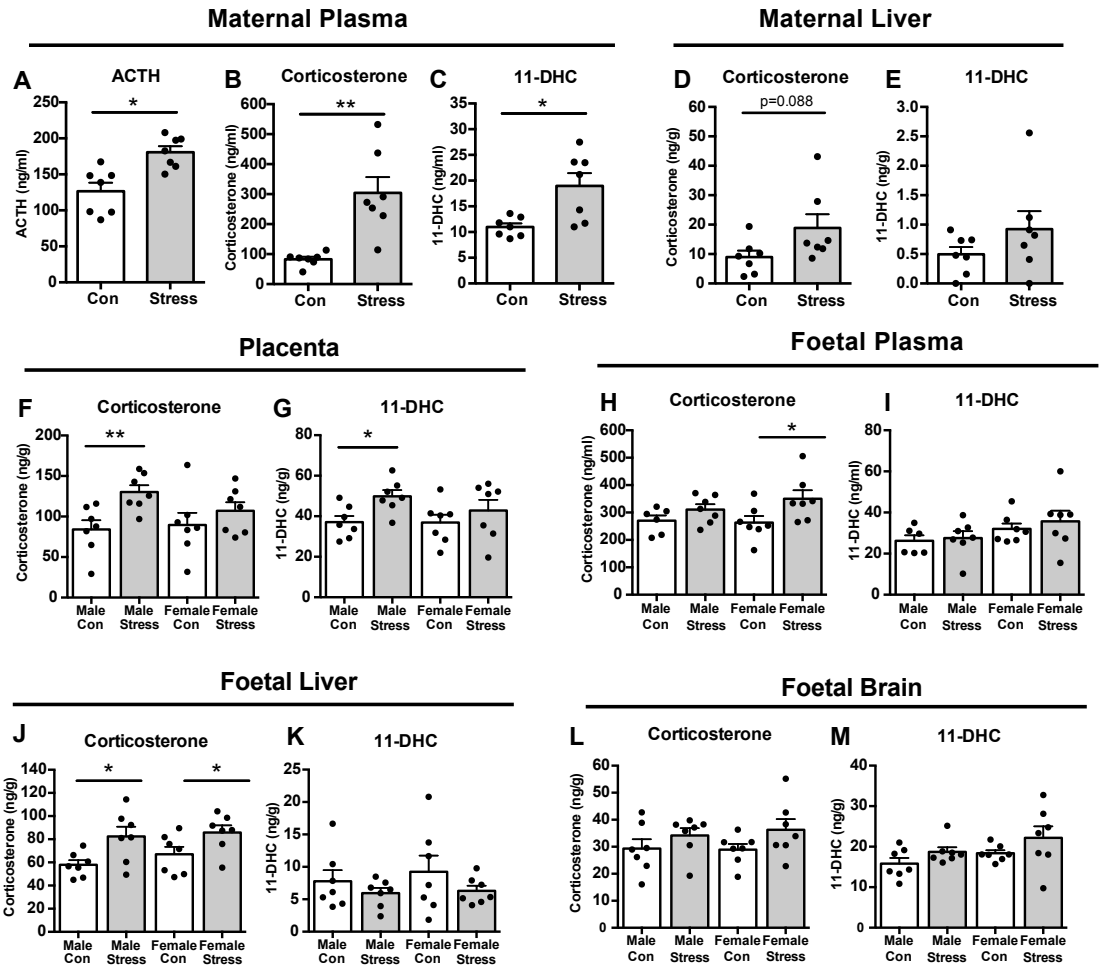


Figure 5.5: Effects of maternal stress on ACTH, corticosterone and 11-DHC concentrations in the mother, placenta and foetuses. Maternal stress resulted in greater plasma ACTH (A), corticosterone (B) and 11-DHC (C) concentrations in the stressed dams, compared to the non-stressed dams (Con). Concentrations of liver corticosterone (D) and 11-DHC (E) were not different between control and stressed dams. Greater concentrations of corticosterone were also observed in the liver of both stressed male and female foetuses (H). Corticosterone concentrations were increased in the foetal liver following gestational stress, in both males (p=0.012) and females (p=0.048). (J) In the foetal plasma, corticosterone was increased following maternal stress in female foetuses, but not in males. There were no changes in the concentrations of 11-DHC in foetal liver (I), plasma (K) or brain (M) following maternal social stress. Asterisks denotes significant differences between control (con) and stress groups, determined by post-hoc SNK pairwise comparisons (where * $p<0.05$, ** $p<0.01$). Note the difference in Y axes ranges, due to the differences in concentrations especially between maternal and foetal compartments, where foetal plasma and liver have greater corticosterone and 11-DHC concentrations than the maternal plasma and liver, respectively.

5.3.2 Placental 11β-HSD2 expression was not compromised by stress

Junctional zone mRNA expression: A main effect of stress was observed in mRNA expression for 11β-HSD2 in the JZ of the placenta ($F_{1,24}$ = 11.7, p=0.0022)(Fig 5.6A and C). Pairwise SNK comparisons revealed that stressed male placenta had significantly higher 11β-HSD2 mRNA expression compared to control male placenta (p=0.004); whilst in females, this difference was not statistically significant (p=0.11). There was no main effect of sex ($F_{1,24}$ = 2.27, p=0.14) nor any interaction between sex and stress ($F_{1,24}$ = 1.16, p=0.29). There were no significant differences between males and females, and post-hoc testing showed that the comparison between the control female and control male group was not significantly different (p=0.08; Fig 5.6A).

Labyrinth zone mRNA expression: In the LB, a two-way ANOVA did not reveal any significance differences between the four groups, and there was no main effect of stress ($F_{1,22}$=1.12, p=0.30), no main effect of sex ($F_{1,22}$=0.778, p=0.38), nor any stress x sex interactions ($F_{1,22}$=0.909, p=0.35) (Fig 5.6B and D).

Protein expression: Western blot detection of 11β-HSD2 protein in placental homogenates (consisting of both junctional and labyrinth layers) did not reveal any significant changes between control and stressed placentae (Fig 5.7A-B; no main effect of stress: $F_{1,24}$=1.64, p=0.21; no main effect of sex: $F_{1,24}$=0.442, p=0.52; no stress x sex interactions: $F_{1,24}$=0.329, p=0.57).

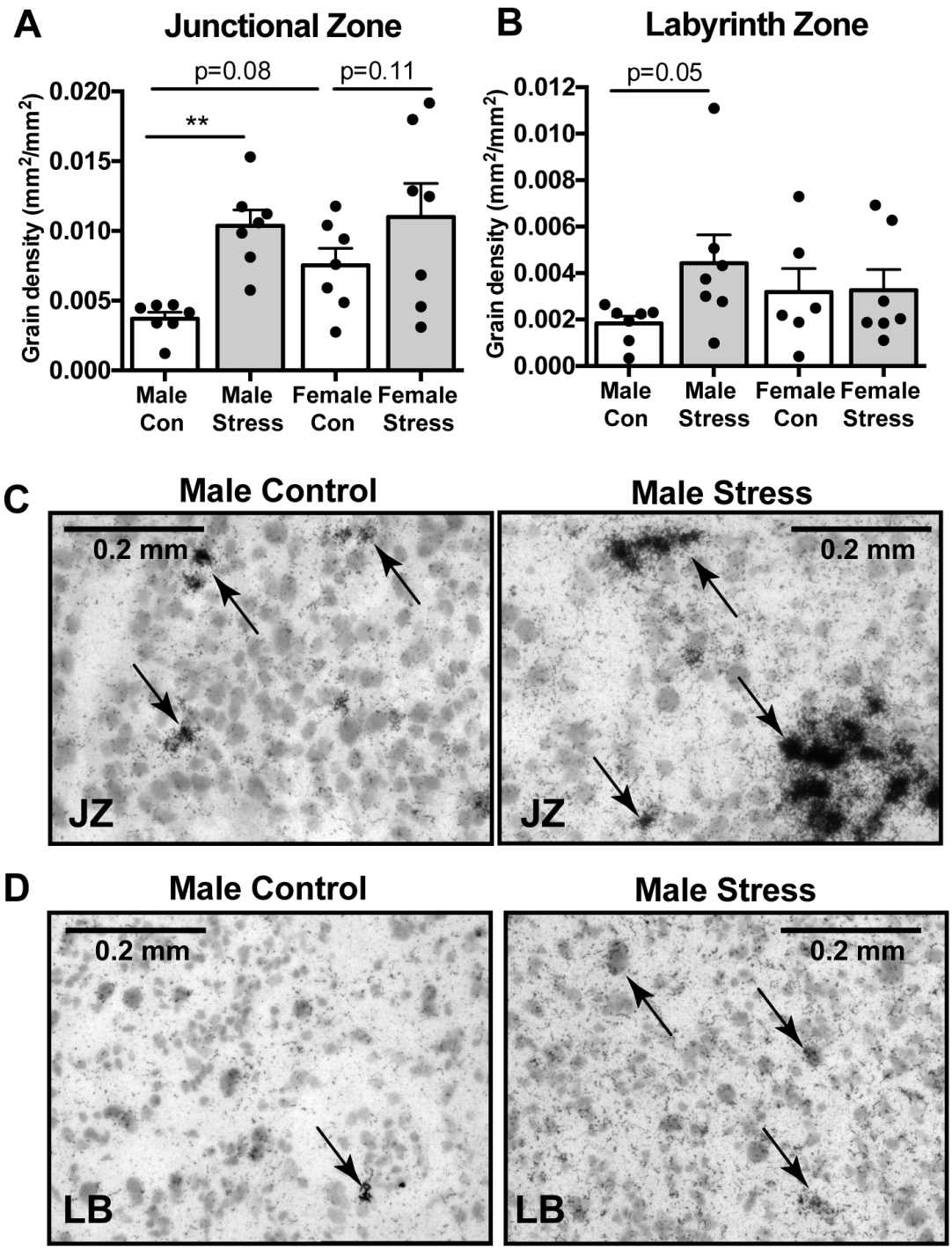


Figure 5.6: Effects of maternal stress on 11β-HSD2 mRNA and protein expression in male and female placenta. In the JZ (A), a significant main effect of stress was observed (two-way ANOVA), and post-hoc SNK testing revealed a significant increase in the placentae from males (p=0.004) following stress, while in females no significant changes were observed (p=0.11). (B) In the LB, a trend towards an increase was observed only in the male stressed placentae. Representative images of 11β-HSD2 mRNA hybridisation in the JZ (C) and the LB (D) of male placenta. Representative images of 11β-HSD2 mRNA hybridisation at the JZ (C) and LB (D) of male control and stressed placentae at 10X magnification.

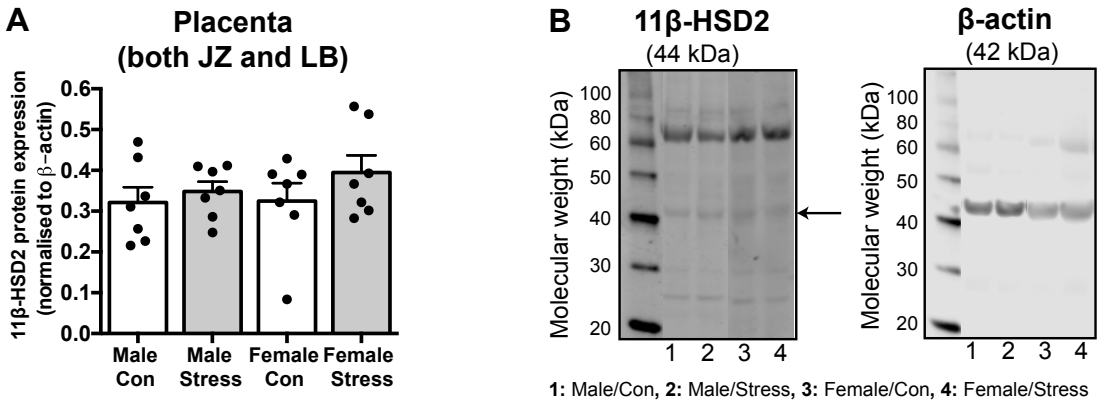


Figure 5.7: Effects of maternal stress on protein expression in male and female placenta. 1/8th of the placenta, consisting of both JZ and LB, was used to quantify 11β-HSD2 protein expression using Western blot. (A) No differences in 11β-HSD2 protein expression were observed between any of the four groups. (B) Representative Western blots of 11β-HSD2 protein (right) and β-actin loading control, after stripping and re-probing (left). Asterisks represent significant differences between control (Con) and stressed groups, where ** p<0.01.

5.3.3 Glucocorticoid sensitivity in the placenta

Glucocorticoid sensitivity of the placenta was investigated by quantifying GR mRNA expression in both the JZ and LB. GR mRNA expression was not significantly different in all four groups in both the JZ (Fig 5.8A-B; no main effect of stress: $F_{1,24}$ = 0.611, p=0.44; no main effect of sex: $F_{1,24}$ = 0.004, p=0.95; no interaction: $F_{1,24}$ = 0.99, p=0.33) and LB (Fig 5.8C-D; no main effect of stress: $F_{1,24}$ = 0.75, p=0.40; no main effect of sex: $F_{1,24}$ = 0.315, p=0.58; no interaction: $F_{1,24}$ = 2.46, p=0.13). No differences were observed between controls and PNS groups, nor between males and females.

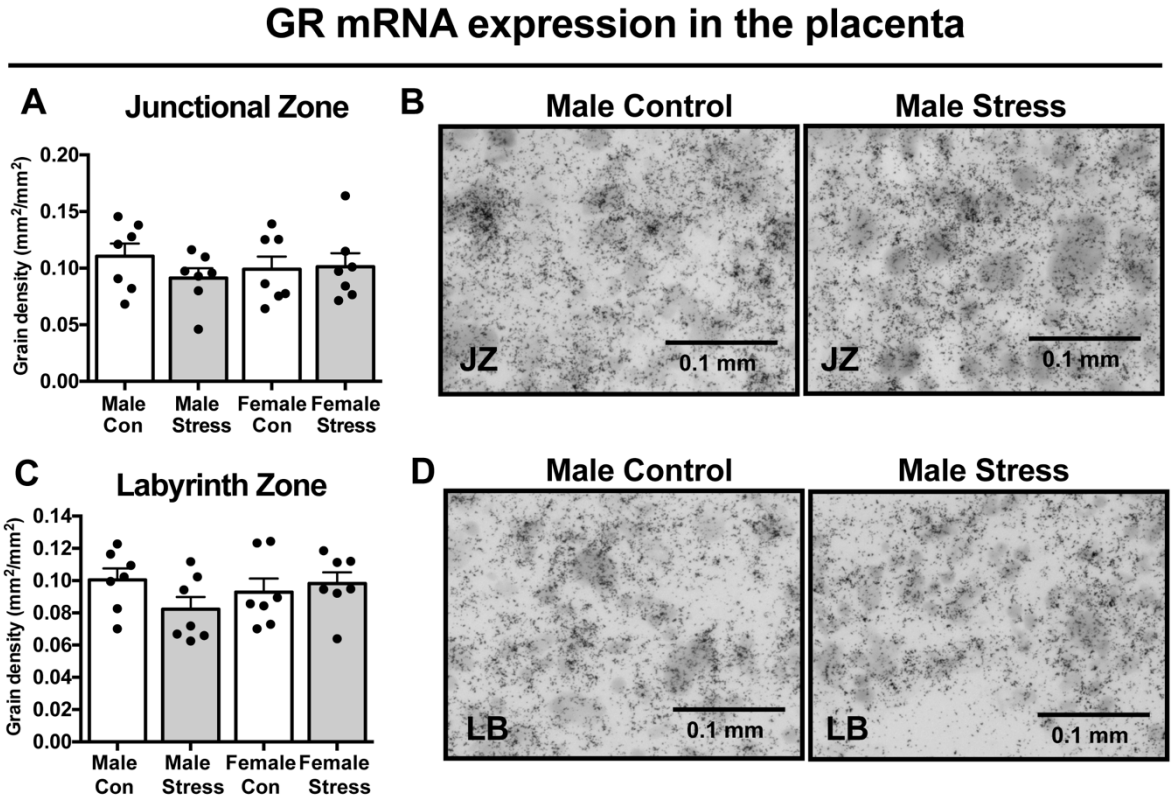


Figure 5.8: Placental GR mRNA expression following maternal stress. GR mRNA expression in the JZ (A) and LB (C) remain unchanged following stress for both male and female placentae. Representative images of GR mRNA hybridisation at the JZ (B) and LB (D) of male control and stressed placentae at 20X magnification.

5.3.4 Glucocorticoid metabolism in the foetal brain

11β-HSD1 and 11β-HSD2 mRNA was also probed for in the foetal hippocampus to determine if there are any changes in local glucocorticoid regulation that might not be revealed with LC-MS detection of steroids in the whole brain. There were however, no significant differences in 11β-HSD1 (Fig 5.9A-B; no main effect of stress: $F_{1,24}$ = 0.911, p=0.35; no main effect of sex: $F_{1,24}$ = 0.0088, p=0.93; no interaction: $F_{1,24}$ = 0.0024, p=0.96) or 11β-HSD2 (Fig 5.9C-D; no main effect of stress: $F_{1,24}$ = 0.951, p=0.34; no main effect of sex: $F_{1,24}$ = 3.44, p=0.077; no interaction: $F_{1,24}$ = 0.31, p=0.58) mRNA expression in the foetal hippocampus in either males or females.

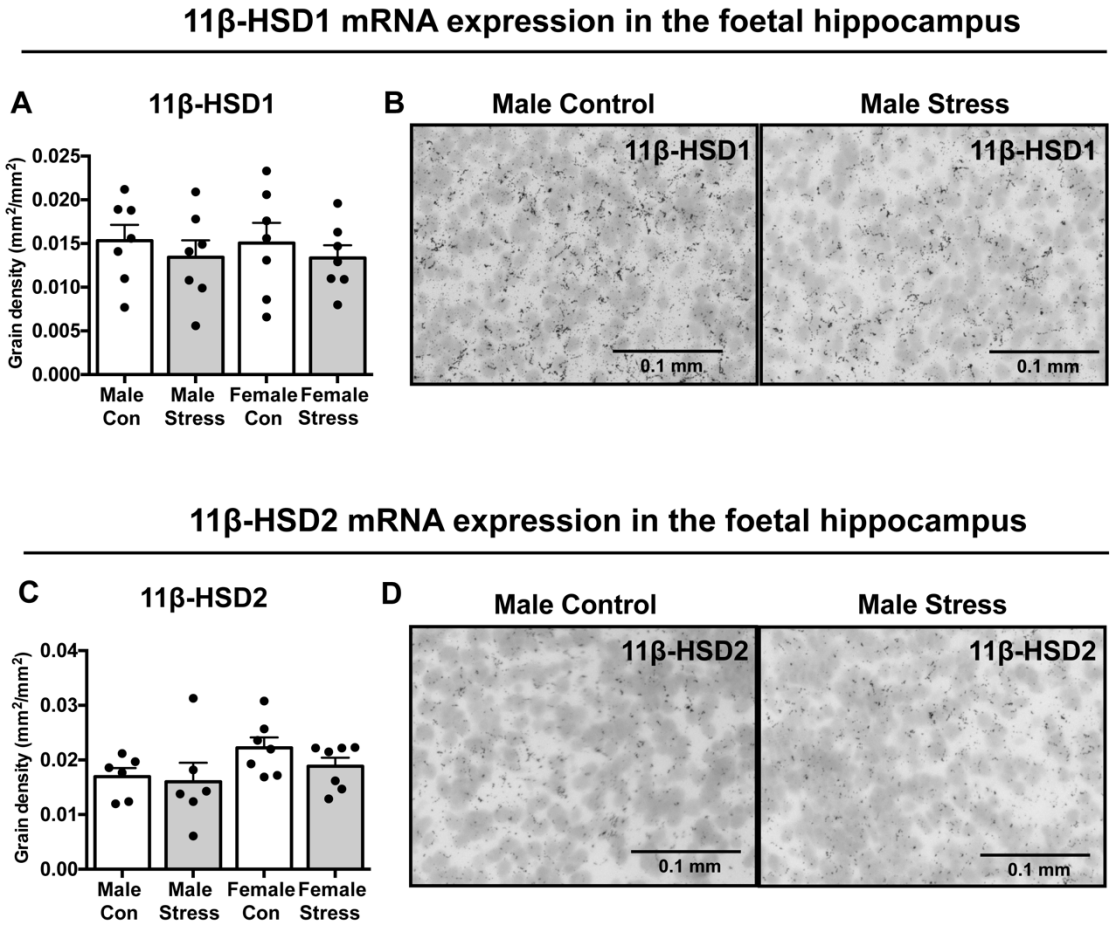


Figure 5.9: Effects of maternal stress on 11β-HSD1 and 11β-HSD2 in the foetal hippocampus. No differences were observed in (A) 11β-HSD1 and (C) 11β-HSD2 mRNA expression in the foetal hippocampus between stressed and control (Con) groups, in either males or females. Representative images of 11β-HSD1 mRNA hybridisation (C) and 11β-HSD2 mRNA hybridisation (D) in the hippocampus of male control and PNS foetuses at 20X magnification.

5.3.5 Effects of stress on steroid concentrations in the placenta and foetuses

Concentrations of progesterone and its metabolites DHP and allopregnanolone, pregnenolone, DOC and its metabolites DHDOC and THDOC, and testosterone were quantified in the placenta, foetal liver and foetal brain.

Progesterone, DHP and allopregnanolone:

Apart from a main effect of stress that was borderline significant for placental progesterone concentrations ($F_{1,24}$=4.28, p=0.05; Fig 5.10A), there were also no effects of sex, nor any stress x sex interactions, on progesterone concentrations in the placenta. Nonetheless, post-hoc testing showed that there were no significant differences among all four groups for placental progesterone concentrations. In the foetal liver (Fig 5.10B) and foetal brain (Fig 5.10C) progesterone concentrations were not significantly different across all four groups. DHP (Fig 5.10D-F) and allopregnanolone (Fig 5.10G-I) concentrations were not different between control and stressed placenta, foetal liver and foetal brain in both sexes. No sex differences were observed as well.

Pregnenolone:

There were no main effects of stress in the placenta, foetal liver nor foetal brain on pregnenolone levels (Fig 5.10J-L). However, in the foetal brain (Fig 5.10L), there was a main effect of sex ($F_{1,24}$=7.39, p=0.012) and the sex x stress interaction was close to significance ($F_{1,24}$=3.31, p=0.081). Post-hoc testing showed that stressed female foetuses had significantly higher brain pregnenolone concentrations compared to control females (p=0.0376), but this difference was not observed in male foetuses (p=0.714). Additionally, the stressed female foetuses also had higher brain pregnenolone concentrations as compared to the stressed male foetuses (p=0.038). There were also main effects of sex for placental pregnenolone on the two-way ANOVA ($F_{1,24}$=5.23, p=0.03), however, post-hoc analyses did not reveal any significant differences between the four groups (Fig 5.10J).

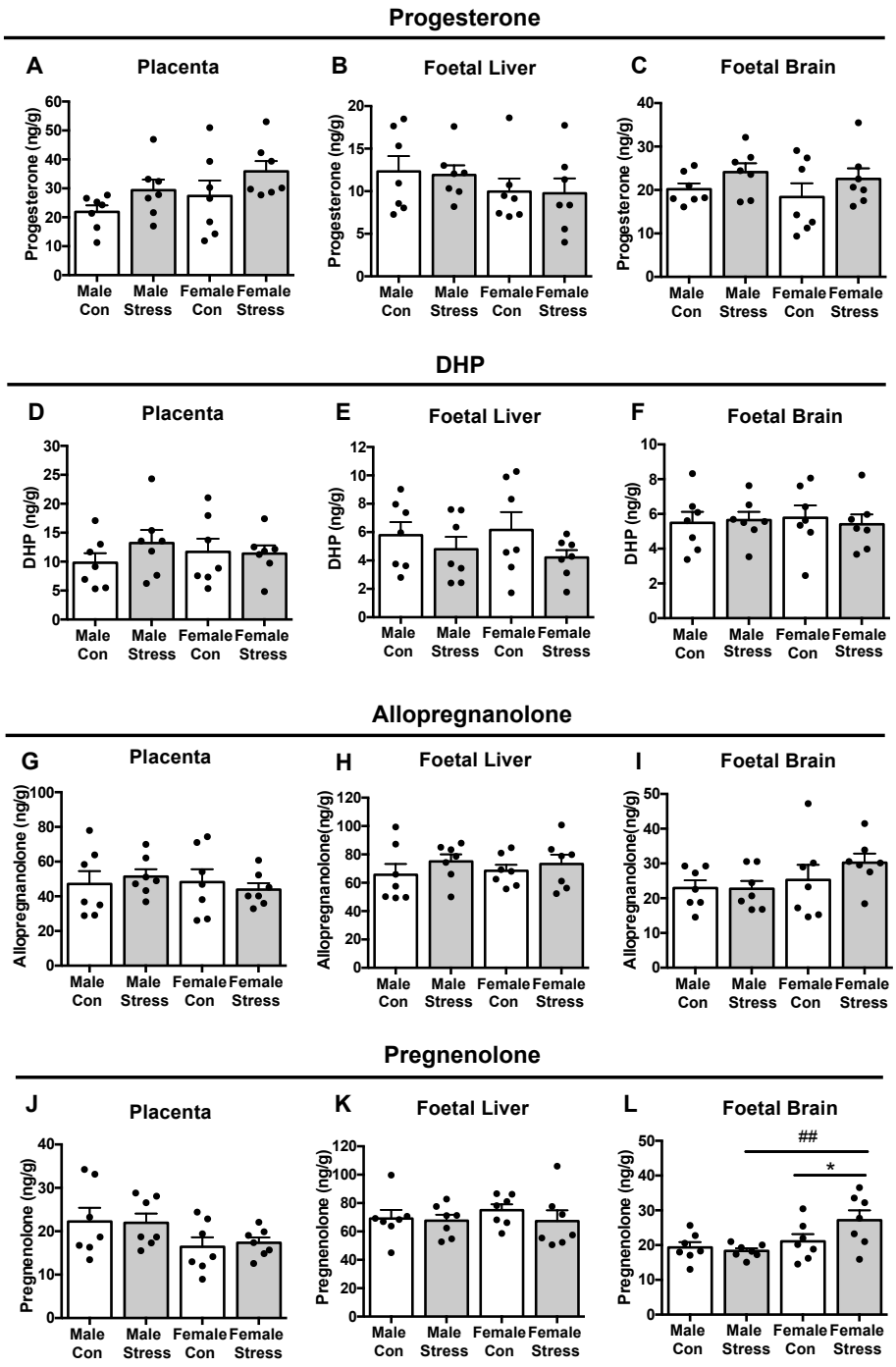


Figure 5.10: Progesterone, DHP, allopregnanolone, and pregnenolone concentrations in the placenta, foetal liver, and foetal brain. Asterisks denotes significant differences between control and PNS groups (where * $p<0.05$) using SNK pairwise comparisons. Hashes denote significant differences between male and female groups (where ## $p<0.01$) using SNK pairwise comparisons. Two-way ANOVA showed a significant main effect of sex on foetal brain pregnenolone. No significant differences in concentrations were observed for other steroids across the four groups.

DOC, DHDOC and THDOC:

There were no significant effects of stress on DOC (Fig 5.11A-C), DHDOC (Fig 5.11D-F) or THDOC (Fig 5.11G-I) concentrations in the placenta, foetal liver nor foetal brain. A main effect of sex, however, was observed for foetal liver THDOC concentrations ($F_{1,24}$=8.03, p=0.009; Fig 5.11H), where post-hoc pairwise comparisons showed control female foetuses had higher liver concentrations of THDOC compared to control male foetuses (p=0.026). A main effect of sex was also observed for foetal brain DHDOC concentrations on the two-way ANOVA ($F_{1,24}$=4.61, p=0.042; Fig 5.11F), however, post-hoc analyses revealed that there were no significant differences between all four groups.

Testosterone:

Lastly, there were no effects of stress on testosterone concentrations in the placenta, foetal liver or foetal brains (Fig 5.11J-L). However, a main effect of sex was observed for testosterone concentrations in the foetal liver ($F_{1,24}$=22.6, p<0.001; Fig 5.11K) and foetal brain ($F_{1,24}$=52.2, p<0.001; Fig 5.11L). There were no main effects of stress nor any interactions. Female foetuses had significantly lower brain and liver testosterone concentrations compared to male foetuses, regardless of stress status.

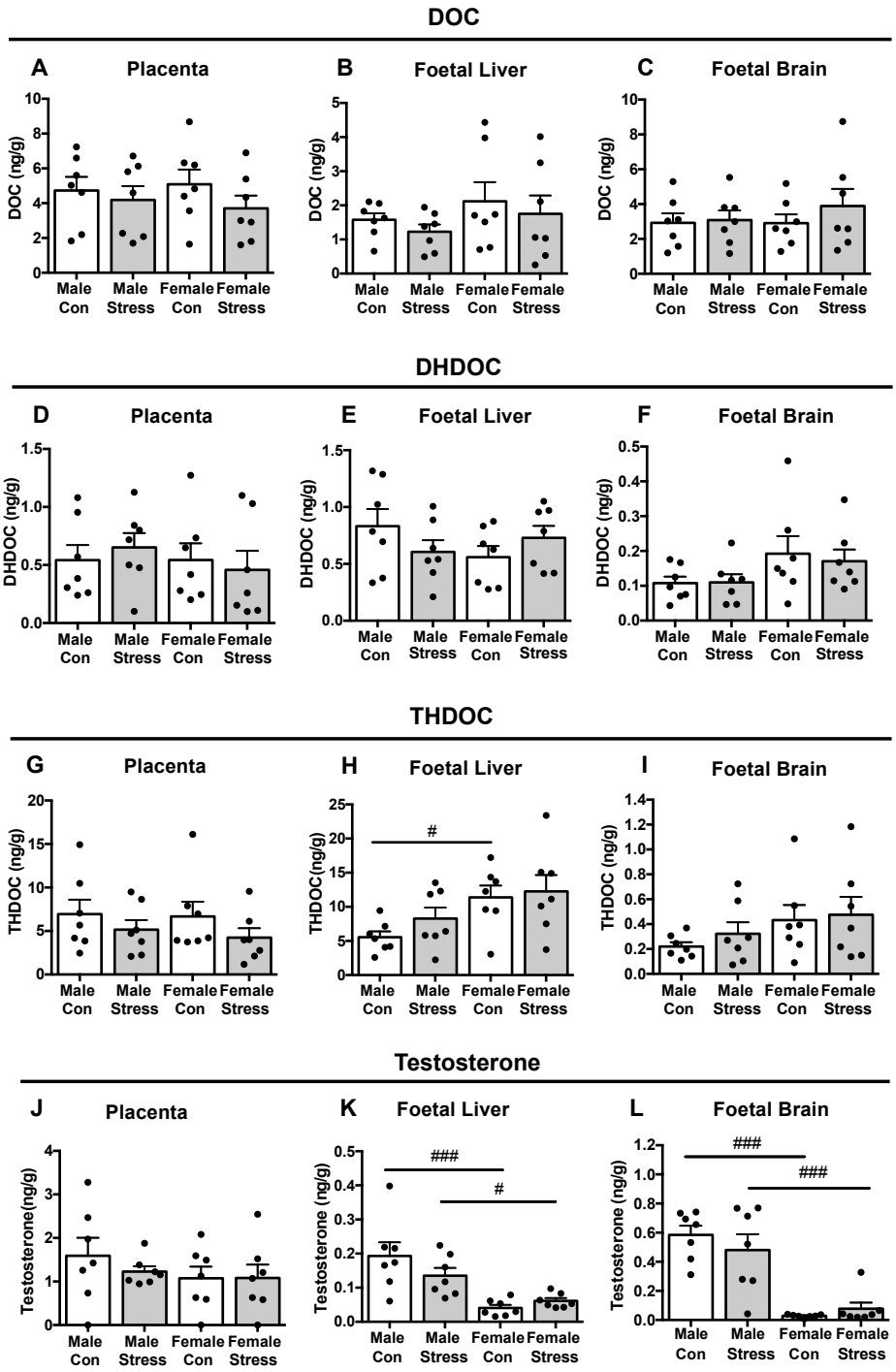


Figure 5.11: DOC, DHDOC, THDOC, and testosterone concentrations in the placenta, foetal liver, and foetal brain. Two way ANOVA revealed that there was a main effect of sex for foetal liver THDOC (H) and a main effect of sex for testosterone in the foetal liver (K) and brain (L). No effect of stress was observed for any of the steroids investigated. Hashes denote significant differences between male and female groups (where # $p<0.05$, ## $p<0.01$, ### $p<0.001$) using SNK pairwise comparisons. Con: Control

5.3.6 Summary of results

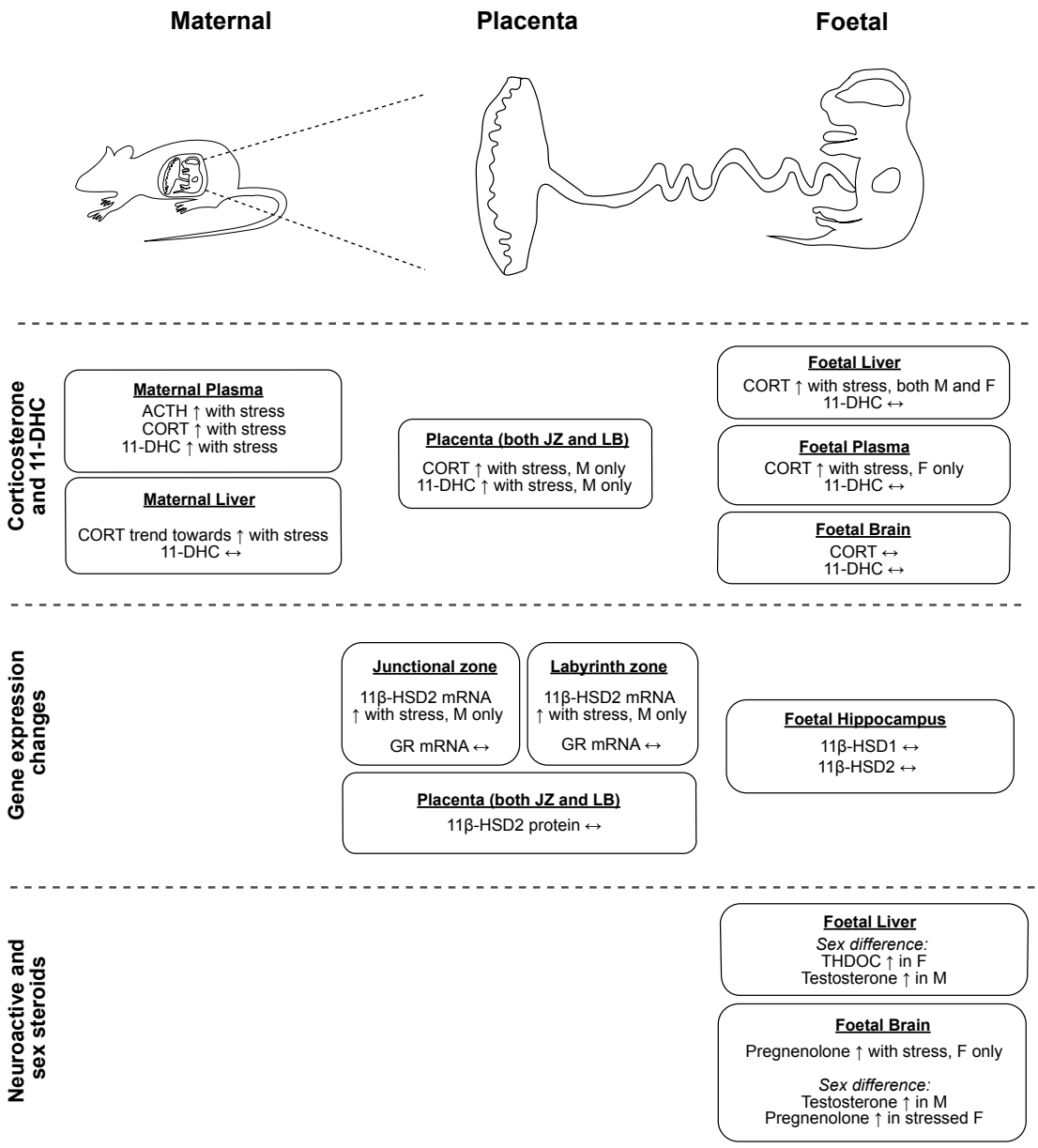


Figure 5.12: Summary of results from Chapter 5. ↑ denotes elevated levels, while ↓ denotes decreased levels, whilst ↔ denotes no difference, compared within each sex by stress status ("with stress"), or by sex (regardless of stress status unless otherwise stated). Abbreviations: M: Male, F: Female, CORT: Corticosterone, 11-DHC: 11-dehydrocorticosterone, DOC: Deoxycorticosterone, DHDOC: Dihydrodeoxycorticosterone, THDOC: Tetrahydrocorticosterone

5.4 DISCUSSION

In this study, pregnant dams were stressed for five days and were killed immediately after the last bout of stress on GD20. Plasma and tissues from pregnant dams, placenta and foetuses were analysed. The primary aim is to test the hypothesis that excessive maternal glucocorticoid crossover, contributed by changes in placental 11β-HSD2, is the main mechanism mediating foetal programming. The second hypothesis is that programming effects could be caused by changes in neuroactive steroid levels following stress. Results are summarised in Fig 5.12, and several key observations refute these two hypotheses.

Firstly, foetal circulatory corticosterone concentrations did not parallel the increase in maternal circulatory corticosterone concentrations, and there was limited trans-placental crossover of corticosterone following the stressor from mother to foetus. There was a minor increase in corticosterone in the foetal liver, but importantly corticosterone changes were not observed in the foetal brain. Placentae from stressed dams expressed significantly greater 11β-HSD2 mRNA in the JZ compared to placentae from control dams, which may imply greater protection by the "glucocorticoid barrier" instead.

Secondly, there were minimal changes in neuroactive steroid concentrations induced by stress in the placenta, foetal liver and brain. Additionally, compared to those observed in the adult offspring, sex differences in foetal tissues were more subtle and only observed for certain steroids.

5.4.1 Changes in maternal and foetal glucocorticoid levels following stress

As expected, there was a significant difference in plasma corticosterone concentrations between stressed and non-stressed dams immediately after social stress at GD20, which is likely to be a result of activation of the maternal HPA axis (Fig 5.5). Despite this robust increase in the maternal circulation, only a modest increase was observed in the plasma of female foetuses, and no change was observed in the male foetuses (Fig 5.5). Additionally, although maternal corticosterone concentrations in the stressed group was three-fold greater than in the control group, foetal corticosterone concentrations in the stressed group was only 1.3-fold greater than the control group, and this was only observed in the female foetuses. The data obtained here corroborates previous findings in mouse

studies where a similar sex difference in foetal corticosterone was observed, with a modest increase in the female foetuses but not in the males (Montano et al., 1993).

One of the tenets of the glucocorticoid programming hypothesis is centred around the assumption that circulating foetal concentrations of glucocorticoids are considerably lower (often, 5- to 10- fold lower) than that of the maternal circulation (reviewed in (Chapman et al., 2013)). Excess glucocorticoids, which are small and lipophilic, would therefore have the tendency to cross over the placenta via simple diffusion, unless inactivated by placental 11β-HSD2. However, contrary to these assumptions, absolute values of foetal circulating corticosterone concentrations were in fact, higher than maternal concentrations (Fig 5.5). At baseline, foetal corticosterone concentrations were three times that of maternal values at GD20 (Fig 5.5), and even following stress, maternal circulating values barely exceeded that of foetal values. Similarly, baseline 11-DHC concentrations were also found to be two-fold greater in the foetal circulation as compared to the maternal circulation and did not exceed foetal values even after stress (Fig 5.5).

These observations, seemingly discrepant from previous assumptions, were possibly due to dynamic corticosterone levels across different gestational stages. When gestational stage is considered, the concentrations obtained in this study are similar to those of previous studies. The values of basal plasma corticosterone in non-stressed rats obtained here using LC-MS (maternal: 82 ng/ml, foetal: 275 ng/ml) were close to the values obtained previously using a fluorometric quantification method (Dupouy et al., 1975) or RIA (Ward and Weisz, 1984), where foetal plasma concentrations were found to be 300-400 ng/ml, whilst maternal concentrations ranged from 100-200 ng/ml on GD19. Even with stress, reported increases in foetal corticosterone were never to the same extent as that seen in the pregnant rat dam (Williams et al., 1999, Takahashi et al., 1998, Bingham et al., 2013). For instance, Williams et al. (1998) showed that stress at GD19 increased maternal corticosterone from 156.1 ng/ml to about 400 ng/ml, whilst foetal concentrations increased from 250 ng/ml to about 310 ng/ml. In light of these ratios, maternal-to-foetal transfer of corticosterone is likely to be complex and does not merely occur via simple diffusion down a concentration gradient. This will be further discussed in the subsequent sections.

In terms of functional significance, it is difficult to conclude whether this modest increase in circulating corticosterone in the female foetuses reported here could have a major influence on foetal growth and development. However, potential implications will be discussed in light of the concentrations in the foetal brain and liver (section 5.4.4), as these are the tissues where the impact of glucocorticoids are particularly relevant.

5.4.2 Changes in placental 11β-HSD2 expression following stress

Sex-dependent changes in placenta 11β-HSD2 mRNA expression were observed following five days of chronic maternal social stress. Male stressed placentae had greater 11β-HSD2 mRNA expression compared to male control placentae, where the difference was significant in the LB and trending towards significance in the JZ. Conversely, 11β-HSD2 mRNA expression was not significantly increased in female stressed placentae as compared to control females, in both the LB and the JZ. Nonetheless, when the 11β-HSD2 mRNA expression is compared between the male and female control groups, there was a trend for female control placentae to have greater expression of 11β-HSD2 mRNA as compared to control males in the LB ($p=0.08$), suggesting that female placentae may have greater baseline expression of 11β-HSD2 mRNA to begin with.

These observations seem to correlate to those observed in humans, where placental 11β-HSD2 is more highly expressed in female placentae than in males (Mericq et al., 2009). In another human study, it also seems that maternal distress can impact placental 11β-HSD2 expression sex-dependently, as maternal distress is associated with increased 11β-HSD2 mRNA levels in male placentae. Although reduced 11β-HSD2 mRNA expression was reported for the female placentae, baseline values were not known, rendering it difficult to directly compare between the sexes (Mina et al., 2015).

Whilst these observations may appear to explain changes in foetal circulatory corticosterone, where the increase in 11β-HSD2 mRNA expression in the stressed male placenta may have prevented excessive glucocorticoid crossover in stressed male foetuses, various clues, discussed in this section and in section 5.4.3 and 5.4.4, suggest that the link between placenta 11β-HSD2 expression and foetal corticosterone levels are not straightforward.

Firstly, it has to be noted that here, 11β-HSD2 mRNA was in fact lowly expressed in the LB (Figure 5.6), the location of exchange between maternal and foetal blood, as compared to the JZ. The regional expression pattern of 11β-HSD2 in the JZ and LB at GD20 in this study was therefore closer to that of a term placenta than that of a GD16 placenta (Waddell et al., 1998). In support, the cellular expression pattern observed also corresponded to that in a term placenta, where there was an intense positive signal for 11β-HSD2 mRNA in some JZ trophoblast cells, but it was completely absent in others (Waddell et al., 1998). Therefore, given the low expression of 11β-HSD2 mRNA in the LB at GD20, it seems that the contribution of 11β-HSD2 on controlling the passage of maternal glucocorticoid to the foetus may not be as integral as once thought.

Secondly, although increased 11β-HSD2 mRNA expression in the LB implies greater conversion of corticosterone to 11-DHC, greater plasma 11-DHC concentrations were not observed in the male foetal circulation. Instead, there was a robust increase in 11-DHC concentrations in the maternal circulation, which could potentially be related the higher expression of 11β-HSD2 in the JZ, given that the JZ is in contact with maternal blood vessels. 11β-HSD2 in the placental JZ thus could have played a role in the converting the excess maternal corticosterone into 11-DHC, which then exited through the maternal circulation instead of entering the foetal blood vessels. The increase in 11β-HSD2 mRNA in placenta during late pregnancy could therefore represent a compensatory mechanism to instead regulate maternal levels of corticosterone that accompanied repeated social stress.

As these mRNA expression changes in 11β-HSD2 are most likely to represent longer term changes induced by five days of chronic stress, there could be other implications. Given that expression patterns of 11β-HSD1 and 2 switch from GD16 to 20 (Fig 5.1), the higher expression in male LB could represent stressed male placentae experiencing a slower reduction in 11β-HSD2 expression as compared to control male placentae during late pregnancy. It is possible that this slower reduction in 11β-HSD2 expression could alter the amount of glucocorticoids they are exposed to basally and during fluctuations of the glucocorticoid circadian/ultradian rhythm, thereby affecting the normal growth trajectory of males.

Despite these changes in mRNA expression for 11β-HSD2, no increase in 11β-HSD2 protein levels could be detected on the Western blot in PNS males. Whilst the

translation of transcribed mRNA into protein is the central dogma of molecular biology, the relationship between mRNA and protein expression have been found to be increasingly dynamic and can be affected by biological factors such as translation efficiency and protein half-life etc (Liu et al., 2016b, Maier et al., 2009). Post-transcriptional modifications is known to occur in human placenta for instance, and pre-eclamptic placenta have different degrees of modifications as compared to placentae in normal pregnancies (Taniguchi et al., 2020). Therefore, it may be possible that gestational stress also altered the post-transcriptional modification of the 11β-HSD2 mRNA, resulting no net changes in 11β-HSD2 protein expression. Additionally, it is worthwhile to note that in this study, an increase in expression of 11β-HSD2 mRNA was only observed in the JZ, both JZ and LB were used for Western blotting detection of proteins, and there is a possibility that it may have masked any differences (if any) in the JZ. In future studies, the placenta can be dissected into the various sub-regions under a dissecting microscope, where the two zones can be differentiated based on their colour, given their different vascularisation characteristics (Ain et al., 2006).

5.4.3 Other mechanisms regulating trans-placental glucocorticoid transfer

The ability to measure 11-DHC concentrations in the maternal and foetal compartments in this study also sheds light on the complexity of the trans-placental glucocorticoid transfer. As mentioned in section 5.4.2, 11-DHC concentrations were increased in the maternal circulation but were unchanged in the foetal compartment following stress, despite changes in 11β-HSD2 mRNA expression.

Recent studies utilising *ex vivo* placental perfusion have revealed that the kinetics of the glucocorticoid diffusion appear to be more complicated than previously thought. In the GD21 rat placenta, the rate of 11β-HSD2-mediated corticosterone to 11-DHC conversion is found to be dependent on concentrations of perfused corticosterone, and there is a limit as to how much 11-DHC could be produced, possibly due to the saturation of enzyme activity (Staud et al., 2006). There also seem to be no differences in characteristics of maternal-to-foetal versus foetal-to-maternal transfer, indicating the possibility of a bi-directional transfer of corticosterone (Staud et al., 2006).

Similar studies on human placenta have additionally revealed that the "glucocorticoid barrier" is not merely contributed by 11β-HSD2. Using near-term

placenta collected from C-section births, when active cortisol was perfused through the maternal blood vessels, a five-fold higher inactive cortisone release was found in the maternal circulation than in the foetal circulation (Stirrat et al., 2018), which seems to correlate to the increased patterns of maternal 11-DHC increase observed in this study following stress, where the converted 11-DHC appears to be returned to the maternal circulation. Additionally, in these perfusion studies, even when 11β-HSD2 is completely inhibited using carbenoxolone, less than 10% of the total injected glucocorticoid actually crosses the placenta into the foetal compartment (Stirrat et al., 2018).

It has thus been suggested that there are several factors other than 11β-HSD2 action controlling the crossover of corticosterone, such as placental vascularity and flow rate of the blood in maternal and foetal vessels, or the role of efflux transporters that regulate active transport of cortisol (e.g. ABC transporters or P-glycoprotein) (Stirrat et al., 2018). It is not known if these other mechanisms can be affected by maternal stress, or if the increase in corticosterone in the foetal plasma of females observed here are due to compromises in any of these other mechanisms. Nonetheless, the ability of the LC-MS method to quantify and compare the levels of corticosterone and its inactivate metabolite 11-DHC may allow for similar perfusion studies in the future.

5.4.4 Changes in the foetal HPA axis following maternal stress

Considering plasma was collected from decapitated foetuses, and not directly from the umbilical vein which would more closely represent trans-placental passage (Fig 5.2), one cannot rule out the possibility that the foetuses' own adrenal glands contribute to circulating foetal corticosterone concentrations following maternal stress. One way to determine this is to measure ACTH levels in the placenta, as ACTH cannot cross the placenta. Hence, an increase in ACTH in the foetuses would indicate activation of the foetal HPA axis by maternal stress and production of corticosterone of foetal origin is highly possible. This was not carried out as the pooled foetal plasma volume was too low to be able to detect foetal ACTH in this experiment.

However, previous studies seem to suggest that whilst single stressors can stimulate an increase in ACTH secretion in the foetus, this is not the case for chronic

stressors, both on a basal level (e.g. a day after the last stressor), and shortly after the last stressor.

Studies utilising a single maternal restraint stress on GD20 have reported that foetal plasma ACTH concentrations are increased, together with foetal corticosterone in stressed rat foetuses (Ohkawa et al., 1991b). Additionally, c-Fos concentrations in the foetal rat PVN are elevated with 30 min after a single 30 min acute stressor (restraint/ immobilisation/ forced walking) on GD21, indicating activation of the brain regions involved in stimulating the HPA axis (Fujioka et al., 2003).

Conversely, in one model of chronic restraint stress (GD11-20) in pregnant rats, basal ACTH concentrations in the foetal plasma were reduced, although this was investigated at GD21, a day after the last stressor (Mairesse et al., 2007). Even when plasma was collected immediately, or up to 90 min after the last stressor, no increase in foetal ACTH concentrations was observed, in the case of chronic variable maternal stress (from GD14-21) during the rat pregnancy (Williams et al., 1999). In the foetal rat brain, PVN c-Fos activation following chronic maternal immobilisation stress from GD17-21 tends to be of lower magnitude compared to that following exposure to a single stressor on GD21 (both investigated 30 min after cessation of a 30 min maternal restraint stress), suggesting that central mechanisms of foetal response to *in utero* stress for chronic stress are different to those for acute stress (Tobe et al., 2005).

5.4.5 Changes in placenta glucocorticoid metabolism and action following maternal stress

The abundance of GR expression in the placenta also suggests that the near-term placenta is itself a prominent target of glucocorticoid action (Mark et al., 2009, Thompson et al., 2002).

Local glucocorticoid content in placenta:

When the content of corticosterone was measured in the placenta, stressed male placentae showed greater corticosterone and 11-DHC concentrations compared to the controls, but this was not observed in the placentae from females. This pattern corresponds to that seen in a mouse study by Montano et al., where there was an increase in the placental corticosterone concentrations in male placentae but not females (Montano et al., 1993). As the placental homogenate does not only

represent intracellular content, it is not possible to determine if the differences are a result of changes in local glucocorticoid conversion (i.e. 11β-HSD1 and 11β-HSD2 action), or if it is contributed by corticosterone and 11-DHC found in the foetal blood vessels and the maternal blood sinuses, derived from foetal and/or maternal sources, or if the glucocorticoids are produced by the placenta's own steroidogenic machinery.

Nonetheless, the values detected in our study correspond to those previously reported in other rat studies, in terms of absolute values (Schmidt et al., 2019b), corticosterone to 11-DHC ratios (Heussner et al., 2016) and placental:maternal corticosterone ratios (Mark et al., 2009). Although tissues from both zones were used for corticosterone quantification here, corticosterone concentrations are not reported to be different between the two zones in the rat placenta (Mark et al., 2009).

Simultaneous quantification of placental 11β-HSD1 mRNA expression could provide more information about the local glucocorticoid conversion processes occurring in the placenta. Far less studies have been carried out to investigate how stress may affect the expression of 11β-HSD1 in the placenta. In a previous study using a rat model, whilst there was no effect of chronic mild stress during GD11-20 on 11β-HSD1 nor 11β-HSD2 protein levels at GD21, higher maternal corticosterone levels was correlated with lower 11β-HSD1 protein levels in the placenta (Lan et al., 2017). Prenatal betamethasone administration in mice on the other hand, does not alter 11β-HSD1 protein expression (Ni et al., 2018). Placental 11β-HSD1 may possibly be further stimulated by glucocorticoids and GR activation in a feed-forward mechanism, although this has only been investigated in the liver of wild-type mice (Morgan et al., 2014). As male placenta had increased corticosterone and 11-DHC concentrations, it is possible that there may also be sex differences in placental 11β-HSD1 expression.

Other factors affecting placental glucocorticoid action:

Ultimately, it is equally important to investigate whether the expression of target receptors are modified following maternal stress, in order to determine functional implications of altered steroid concentrations. GR mRNA expression patterns observed in this study were similar to what has been described in previously published studies in the rat, where GR was present in both the JZ and the LB (Heller

et al., 1986, Waddell et al., 1998). In this study, gestational stress did not lead to differences in GR mRNA expression in control versus stressed placentae, either n the JZ or in the LB, indicating that the number of GRs are unlikely to be altered by gestational stress.

However, it is still not possible to conclude if there were any differences in glucocorticoid action on GR, as glucocorticoid action not only depends on the density of GR, but also on their phosphorylation status, isoform expression, and the number or the function of chaperone proteins they are associated with, to name a few (Oakley and Cidlowski, 2013, Scheschowitsch et al., 2017). For instance, sex-dependent alterations in the expression of GR isoforms is observed following maternal dexamethasone exposure in the mouse, leading to impairment of placental development (Cuffe et al., 2017b). Sex difference in glucocorticoid resistance have been postulated to occur through differential expression of various GR isoforms in the human placenta (Saif et al., 2014). These GR isoforms arise through splice variants, which were not considered for the GR riboprobe used here, which targets sequences corresponding to the GR steroid-binding-domain (riboprobe sequence first published in (Diaz et al., 1998)).

Additionally, it is not known if chaperone proteins associated with GR, such as FKBP51 or FKBP52 which are also expressed in the placenta (Tranguch et al., 2007), may be altered by maternal stress. In humans, maternal stress during the second trimester decreased placental FKBP51 mRNA only in female foetuses (Togher et al., 2018), while placental FKBP52 protein expression seem to be disrupted in pre-eclamptic and IUGR pregnancies (Acar and Ustunel, 2015).

5.4.6 Foetal exposure to glucocorticoids following stress: Foetal liver and brain

Despite glucocorticoid changes in the female foetal circulation, ultimately, the physiological effects of glucocorticoids in the foetus are dependent on their action in the target organs.

Foetal liver:

Greater hepatic corticosterone concentrations were observed for stressed foetuses, regardless of sex. Given that the umbilical vein directly drains into the foetal liver (Fig 5.2), the foetal liver is the first organ to be perfused by placental blood (Murphy,

2005, Lautt, 2009). The majority of the blood bypasses the liver via the ductus venosus into the inferior vena cava and the rest of the body, however, a proportion of it perfuses the liver. At the same time, the liver is also perfused by the hepatic portal vein, where corticosterone produced by the foetal adrenal glands could be transported into the liver (Murphy, 2005, Lautt, 2009). As it is not possible to differentiate between the contributions of these different sources of corticosterone, the observed results in this study could represent intracellular corticosterone content in liver tissue, corticosterone in the blood from the umbilical vein, as well as from the production from the foetal adrenal glands.

This also the first time foetal and maternal corticosterone are compared in the same study, possibly due to prior constraints in measuring liver glucocorticoids using RIA. Here, it is observed that foetal hepatic glucocorticoid content is far greater than that in the maternal liver. Although the reason for this is not known, it is likely to be related to the glucocorticoid surge in foetuses that occurs at late pregnancy. Additionally, there are also dynamic changes in expression of CBG mRNA through gestation, where foetal CBG mRNA expression is greater compared to maternal concentrations at GD15, but decreases to a low level at birth (Smith and Hammond, 1991, Leeper et al., 1988). Although foetal liver glucocorticoids were measured at GD20 in this study, which again represents a snapshot instead of the dynamic changes that are occurring in late pregnancy, there is a possibility that hepatic CBG have further aided the sequestering of corticosterone in the foetal liver. The high concentrations of hepatic corticosterone in the foetus is likely required for the functional maturation of the liver, as the glucocorticoid surge is accompanied by increased glucose production and glucogenic proteins (Hyatt et al., 2008).

The implications of altered liver corticosterone in the foetus is not known, but this difference could presumably affect the typical growth trajectory or function of the liver at GD20. Previous studies have observed that gestational stress, with increased maternal corticosterone levels, affects hepatic gluconeogenic capacity in rat foetuses, potentially leading to the programming of metabolic diseases (Franko et al., 2017). Using the same social stress model, it was found that there were changes in the expression of genes regulating glucose-insulin homeostasis and lipid metabolism in the offspring during adulthood (e.g. Pgc1a) (Brunton et al., 2013). Similar changes in Pgca1a are also observed in adult rats that were exposed to dexamethasone prenatally (Drake et al., 2010). Although it is not known if foetal liver

corticosterone concentrations were altered in these previous experiments, these studies suggest that processes related to hepatic lipid metabolism seem particularly sensitive to changes in glucocorticoid metabolism and action, and may be associated with changes in GR protein expression for example (Maeyama et al., 2015).

Additionally, given the peculiar way in which the foetal liver is perfused (Fig 5.2), it is also not known if liver perfusion and blood flow is affected by stress. In human pregnancies, under circumstances of reduced oxygen or nutrition, a higher proportion of blood from the umbilical vein bypasses the liver and is directed preferentially to other parts of the body (e.g. the brain) via the ductus venosus (Godfrey et al., 2012). Whether or not this process occurs in this model of social stress in the rat, and whether an alteration of blood flow (if any) would affect the transport of glucocorticoids in the blood and the action of glucocorticoids on the liver and other target organs, is unclear. As the first foetal organ to be exposed to placental blood, the foetal liver could have an important role to play in determining the circulatory glucocorticoid and steroid concentrations in the foetus.

Although not investigated in this study, the liver also expresses 11β-HSD1, and stress could have affected the activity of hepatic 11β-HSD1, leading to changes in corticosterone metabolism in the liver itself. In sheep, exposure to a synthetic glucocorticoid results in elevated hepatic CBG and 11β-HSD1 expression in the foetal liver (Sloboda et al., 2002), while in rats, prenatal stress tends to increase foetal hepatic 11β-HSD1 mRNA levels (Mairesse et al., 2007). In another study, restraint stress was also shown to increase 11β-HSD1 mRNA in the GD18 mouse liver, and this effect persisted through adolescence, where differences manifested as increased 11β-HSD1 protein concentration coupled with an increase in GR for the stressed groups (Maeyama et al., 2015). Whilst 11-DHC:corticosterone ratio were not significantly altered following stress, changes in 11β-HSD1 expression in the foetal liver may still occur, and may contribute towards the development of metabolic dysregulation in adulthood. Previous studies have shown that 11β-HSD1 overexpression in transgenic mice results in insulin resistance and hypertension (Paterson et al., 2004), while liver-specific 11β-HSD1 KO mice seem to be protected from the effects of glucocorticoids and do not develop negative metabolic phenotypes (Harno et al., 2013a). Therefore, an investigation into foetal 11β-HSD or

other steroidogenic enzymes to determine if the steroidogenic capacity of the foetal liver changes with maternal stress could be a future line of work.

Foetal brain:

No significant changes were found in corticosterone concentrations in the foetal brain. As target tissue availability of corticosterone is controlled by uptake from the circulation, the levels of plasma proteins such as CBG and albumin could have contributed to this observation, especially in female foetuses. This has not been studied in the rat, but prenatally stressed piglets indeed showed increased plasma CBG concentrations at the third postnatal day (Kanitz et al., 2003), which could imply reduced glucocorticoid uptake in target tissues.

Additionally, in this study, 11β-HSD1 and 11β-HSD2 mRNA were unchanged with stress in the foetal hippocampus, which indicates that long-term changes in local glucocorticoid metabolism did not occur in the foetal brain. Nevertheless, from our *in situ* hybridisation results and those previously reported (Diaz et al., 1998, Moisan et al., 1992), the expression patterns of 11β-HSD1 and 11β-HSD2 seemed to be quite low in the foetal brain at GD20 even for control animals, therefore it raises the question of whether 11β-HSD enzymes in the foetal brain play a significant role in modulating brain development during late pregnancy.

In brain-specific 11β-HSD2 KO mice, foetal brain corticosterone content was not different from controls (Wyrwoll et al., 2015). Expression of 11β-HSD2 in the brain however, was also found to be extremely low in controls on GD17.5 to begin with. Moreover, in adulthood, these 11β-HSD2 KO mice do not show anxiety-like behaviour, but only depressive-like behaviour. Considering that anxiety is also one of the hallmark phenotypes observed in adult offspring in the prenatal social stress model, our data supports their contention that 11β-HSD2 in the foetal brain perhaps does not play an important role in the programming of anxiety behaviour (Wyrwoll et al., 2015).

Whilst a brain-specific 11β-HSD1 KO mouse model is available (Harno et al., 2013b), little has been studied about its effect on offspring behavioural dysregulation. As mentioned earlier, there is also a need to investigate the expression levels of target receptors of glucocorticoid action such as GR and MR (Diaz et al., 1998) in order to obtain a clearer picture of glucocorticoid metabolism in

the foetal brain. The investigation into other components of the HPA axis in the foetal brain would also be practical, as stress seemed to be able to also change expression of these indices, such as CRH mRNA in the PVN of rat foetuses (Fujioka et al., 1999).

To summarise this section, whilst corticosterone could play a role in the programming of the foetal liver following stress, it probably does not play a direct role in determining programming outcomes in the foetal brain.

5.4.7 Other changes in the maternal compartment following chronic stress

Maternal liver glucocorticoid metabolism:

In the maternal liver, there was only a trend towards greater corticosterone and 11-DHC concentrations in stressed livers as compared to non-stressed livers, despite robust increases in the maternal circulation. Again, concrete conclusions cannot be drawn about whether this was an effect of chronic or acute stress, as there was no comparison to a pregnant rat that has only been through one stressor. Moreover, it is recently found in mouse studies that the maternal liver exhibits dramatically reduced glucocorticoid signalling and responsiveness during pregnancy, with hepatic GR downregulation via epigenetic silencing (Quinn et al., 2019). As excessive glucocorticoid action can impair growth, this adaptation has been proposed to temporarily increase maternal liver growth to meet the metabolic demands of pregnancy. Hence, given that the maternal hepatic corticosterone and 11-DHC only showed a trend towards an increase, it is possible that the pregnant liver may be actively metabolising excess corticosterone produced from the maternal adrenal glands, as a compensatory mechanism to avoid increased glucocorticoid signalling in the liver, which may be pathogenic. If chronic stress were to disrupt this process, it could lead to metabolic imbalances during pregnancy, promoting the risk of IUGR (Quinn et al., 2019). This also brings about the idea that changes in glucocorticoid metabolism following stress is global in the mother, and that aberrant glucocorticoid signalling in other maternal organs during pregnancy may conceivably also lead to aberrant foetal outcomes.

Impacts on maternal health:

Although maternal glucocorticoid metabolism was investigated in GD20, there is a possibility that these changes following chronic social stress may result in long-term

changes in the maternal HPA axis beyond the pregnancy. Repeated stress exposure during pregnancy is also known to result in elevated levels of corticosterone during the postnatal period in rat dams (Pfister and Muir, 1989) and a decreased GR expression in CA3 of the hippocampus of the pregnant rat (Pawluski et al., 2015). As 11β-HSD1 expression in the PVN usually increases towards the end of pregnancy (Johnstone et al., 2000), there is a possibility repeated stress can abrogate the normal increase, leading to problems with HPA axis negative feedback inhibition. These longer-term changes may have implications such as poorer maternal care, as well as a higher risk for post-partum mood disorders, evident from rat studies where stress induces depressive-like behaviours in dams post-partum (Smith et al., 2004, O'Mahony et al., 2006), especially because the period immediately after parturition is a window of vulnerability where there is a dramatic alteration in various hormonal systems (Bosch et al., 2007, Slattery and Neumann, 2008). Although not a focus of this study, the possible impacts on maternal health is still a much needed area of research, as not only does it affect the well-being of the mother, in the context of this thesis, maternal behaviour during the postnatal period is also one of the mechanisms mediating the outcomes of prenatal stress (section 1.5.4).

5.4.8 Changes in neuroactive steroids concentrations in the foetal brain and liver following stress

In general, absolute concentrations of neuroactive steroids were much higher in the foetal rat brain as compared to adult values obtained in Chapter 4, as expected (Brunton et al., 2014). This was also the first time concentrations of GABAergic neuroactive steroids such as allopregnanolone and THDOC were determined in the foetal brain, whilst concentrations of its precursors progesterone and DOC corresponded with that shown in a recently published LC-MS study (Schmidt et al., 2019b). However, previous studies did not quantify the steroids based on foetal sex.

Allopregnanolone:

Placental, foetal brain, and liver allopregnanolone concentrations were not different between the stressed and non-stressed groups. In the foetal brain, there was no evidence of an insult-mediated increase in allopregnanolone protection, unlike that observed in the foetal sheep (Nguyen et al., 2003a, Nguyen et al., 2004), nor was there a no loss of allopregnanolone protection, unlike that observed following

umbilical occlusion in guinea pigs foetuses (Bennett et al., 2013). As the normal response to an acute stressor in rat foetuses during late pregnancy has not been characterised, it was not possible to determine if these observations were confounded by the effects of chronic stress. Additional measurements of steroidogenic enzyme expression in the foetal brain could perhaps shed light on this.

Pregnenolone:

Pregnenolone concentrations were increased only in the stressed female foetal brain but not in the males. This raises the question of whether or not the increase in pregnenolone was responsible for mediating some of the sex differences in behavioural and physiological outcomes in the PNS offspring in the social stress model (i.e. PNS males show greater anxiety behaviour, whereas PNS females do not, but PNS females tend to show greater HPA axis dysregultionthan the PNS males). Again, it is not known if this observation is confounded by chronic stress, nor the source of pregnenolone, as quantification of p450scc expression in the foetal brain was not carried out. In terms of functional significance, pregnenolone could be sulfated into PregS, which has neuroprotective effects via the NMDA receptor, enhancing neurite outgrowth and neuronal survival (Xu et al., 2012). Sulfotransfereases mRNA are expressed in both human and rat brain foetal brain (Suiko et al., 2017), thus the higher pregnenolone in female brains could possible be converted to PregS, providing better protection against insults by promoting neuronal survival.

DOC, DHDOC and THDOC:

As for the neuroactive DOC metabolites DHDOC and THDOC, stress did not affect concentrations in the foetal brain and liver. Unlike progesterone, DHP and allopregnanolone, the concentrations of DOC, DHDOC and THDOC were largely similar to those found in the adult. A modest sex difference was observed in these steroid concentrations, which will be discussed in the next section. Although these steroids are neuroactive, the exact role of DOC metabolites in modulating foetal growth is not known. It has been suggested that the role of DOC metabolites only increases in the postnatal stages, where there could be a switch from neuroprotection via allopregnanolone alone, to neuroprotection mediated by both allopregnanolone and THDOC, utilising the DOC precursor from the neonatal

adrenal glands (Hirst et al., 2008). Nonetheless, one point to note is that as the reduction of DOC into its metabolites DHDOC and THDOC occur via similar pathways, utilising the same steroidogenic enzymes 5α-reductase and 3α-HSD. The observation that they are present in the foetal brain and liver, albeit in smaller amounts, could mean that the effects seen in earlier experiments involving 5α-reductase inhibition and effects on prenatal stress outcomes need to be re-interpreted with caution (Paris et al., 2011). Since finasteride blocks the reduction of all 5α-reduced steroids, it will effectively affect several hormonal systems (e.g. progesterone, DOC and even testosterone metabolism pathways), thus the effects of such a blockade cannot be attributed to the loss of allopregnanolone actions alone.

To conclude, similar to the effects of glucocorticoids in the foetal brain, there is probably a limited direct effect of steroidal changes in the programming of brain development. Nonetheless, comparisons with acutely-stressed foetuses would be helpful, as the steroidal response of rat foetuses to a single acute stressor during late pregnancy has not yet been established.

5.4.9 Sex differences in the steroidal milieu during late pregnancy

In Chapter 4, it was determined that there was a robust sex difference in the absolute amount of each neuroactive steroid in the brain of adult male and female rats. Here, at GD20, sex differences in neuroactive steroids in the brain were generally not observed, except for testosterone, where a significant difference was found between male and female foetal brain. A similar difference was also observed for testosterone in the foetal liver. Concentrations of progesterone and its metabolites were not different between males and females, whilst there were modest sex differences in THDOC content in foetal liver.

Testosterone:

It is known that plasma testosterone concentrations in the rat are different between male and female foetuses in late pregnancy, especially during GD17-19 where the testosterone surge occurs in males (Ward et al., 2003). Here, foetal plasma testosterone could not quantified due to the low amounts of foetal plasma collected, however, testosterone levels were found to be significantly greater in males as compared to females in the foetal liver and foetal brain. This is in contrast with the

results obtained using RIA (Kellogg and Frye, 1999, Konkle and McCarthy, 2011) which reported that females and male rat foetuses did not show differences in testosterone concentrations in the brain. However, these discrepancies could probably be attributed to inaccuracies in quantification using the testosterone RIA kit, as one of these studies also oddly reported no differences in adult concentrations of testosterone between males and females (Konkle and McCarthy, 2011), which was not the case given our results in Chapter 4, and that of other studies using validated LC-MS methods (Hojo and Kawato, 2018, Caruso et al., 2013). Absolute concentrations of testosterone obtained by these RIA studies also did not correspond to those obtained using LC-MS, and values were up to a hundred-fold higher, indicating cross-reactivity or an inappropriate enhancement of signal due to interferences in the testosterone RIA, when applied to foetal brain samples. This again highlights the need to carry out steroid quantification studies using improved and validated methods, especially in tissues like the brain, which contains high levels of interfering substances like lipids.

Here, although stress did not change concentrations of testosterone in any of the tissues, differential testosterone concentrations between males and females may still have implications, due to the “organisational” effects of testosterone (Chapter 1), by its interaction with other mediators of the stress response (Goel and Bale, 2009),. For instance, testosterone can enhance serotonergic signalling (Goel et al., 2011) and is important for controlling the expression of androgen receptors (Bingham and Viau, 2008), both of which may influence HPA axis activity (Chen et al., 2016, Goel et al., 2011). Indirect effects of testosterone can also arise from action of its downstream metabolite DHT (Wilson, 2001), as well as the actions of oestradiol (McCarthy, 2008), which is critical for the development of the neurocircuitry which typifies masculinisation of the brain (Wu et al., 2009). Although concentrations of DHT or oestradiol were not investigated here, there is a possibility that their levels can be perturbed, especially since expression of 5α-reductase isoform 1 (Ordyan and Pivina, 2005) and aromatase activity (Weisz et al., 1982) in rat brain foetuses has been shown to be affected by prenatal stress.

Progesterone:

Similarly, previous studies have established that progesterone concentrations do not differ between males and females foetuses, possibly due to the overwhelming

amounts and importance of progesterone during pregnancy (Weisz and Ward, 1980). It was also shown in this study that DHP and allopregnanolone do not differ in the male and female foetal brain, unlike that of adult rats observed in Chapter 4. However, progesterone could exert its sex specific effects in another way, through differential progesterone receptor (PR) expression for example. PR density in the sexually dimorphic medial preoptic area (mPOA) is high in foetal males, while they are virtually absent in the females (Wagner et al., 1998), which has implications in sexual differentiation.

THDOC:

Control female foetuses had greater THDOC concentrations in the liver as compared to control male foetuses, but no differences were observed in the brain. It is not known if these differences are due to differential expression of steroidogenic enzymes between males and females. Whilst 5α-reductase 2 is only expressed in the rat brain during late foetal and early postnatal life, and could be induced or modulated by the testosterone surge in male foetuses, 5α-reductase 1 is constitutively expressed in the rat brain and liver at all stages of brain development and do not seem to show a sex difference (Melcangi et al., 1998). In human studies, an array of steroidogenic enzymes in the foetal liver has been characterised and notably, 5α-reductase 3 (SRD5A3) was found to be differentially expressed in female foetal liver, where a lower expression was seen (O'Shaughnessy et al., 2013). Although this isoform has not been studied in the rat, it supports the contention that sex differences in steroidogenesis can also arise from within the foetal liver itself.

Placental sex differences:

No differences in sex steroid concentrations were observed in this study between the placentae of males and females, possibly due to their central role in maintaining critical aspects of the pregnancy such as placental vasculature (Maliqueo et al., 2016). Nonetheless, the sex difference in placenta is likely to manifest in other ways (Bale, 2011), as seen in the differential 11β-HSD2 mRNA expression earlier in this study, for instance.

Although testosterone content was not different in male and female placenta, there are differences in the expression levels of steroidogenic enzymes that controls the

synthesis of androgens into the foetal compartment, observed in human studies (O'Shaughnessy et al., 2019) and rodent studies alike (Howerton and Bale, 2014). The placenta can therefore contribute to the differential steroid milieu between female and male foetuses and play a role in the sexual differentiation of male foetuses.

5.4.10 Limitations

A major limitation recurring in this study is the inability to differentiate between the effects of acute stress or chronic stress, as immediate effects of the acute stressor at GD20 has been superimposed on the possibly longer-term effects of the chronic stressor (applied from GD16-20). A way to tease this apart is to introduce an additional group of pregnant dams which undergo only one single stressor at GD20, to determine the acute response at that stage. Differences could then be inferred to be the outcome of prior chronic stress (e.g. sensitisation to the stressor, or long term changes). It may also be worthwhile to carry out the tissue collection at GD21, 24 hours after the last chronic stressor, which removes the confounding effects of the acute stressor but at the same time, allows for the determination of the basal level of steroids.

Secondly, the placenta, foetal liver and foetal brain tissues analysed here were not from the same feto-placental unit, while the foetal blood has been pooled. It is difficult to draw concrete conclusions about secretion patterns of the placenta into the foetal plasma or uptake of steroids from the peripheral circulation into the foetal brain and liver, as individual correlations could not be made. This is especially so when the differential intrauterine position is known to introduce considerable variability in steroid concentrations in litter-bearing animals such as rodents (Ryan and Vandenbergh, 2002). For instance, female foetuses located between two male foetuses have a slightly different hormonal milieu than those which are not, and generally exhibit greater testosterone concentrations. Intrauterine positions have been found to affect the outcome of prenatal stress, affecting certain postnatal characteristics in female mice (vom Saal et al., 1990).

5.4.11 Conclusions and future work

In conclusion, this chapter showed that maternal corticosterone does not play a direct role in the transmission of stress signals from mother to foetus, as there were

only small increases in corticosterone concentrations observed in the foetal compartment. In addition, neuroactive steroid protection did not appear to be compromised, in the maternal, placental and foetal compartments. This was the first time the effects of repeated maternal social stress has been investigated in the pregnant dam, placenta and the offspring at GD20, as previous studies with this prenatal social stress model have always been focussed on the effects of offspring during adulthood.

Limitations have been highlighted in the preceding section. In order to add weight to the results observed in this study, *ex vivo* experiments can be designed, where *ex vivo* organ culture of either the entire rat placenta, or large pieces of the human placenta could be used. This has been employed by numerous studies of human placenta to study endocrine activity, the advantage being that cellular elements are maintained in their normal state of organisation (Ringler and Strauss, 1990). Manipulations, such as exposing placental organ cultures to corticosterone, can then be carried out. Media could be collected to determine whether there are any differences in the secretion of steroid metabolites, whilst placenta can be sectioned to determine if mRNA changes still occur in a similar manner as the *in vivo* results. To study the role of the placenta in trans-placental transport, or the synthesis and metabolism of compounds thoroughly, a perfusion system can also be implemented, however, this requires extensive expertise (Goeden and Bonnin, 2013).

Ultimately, as per the allostasis framework, it is necessary to acknowledge that corticosterone concentrations and 11β-HSD2 expression represent only a small aspect of an integrated physiological system regulating the impact of glucocorticoid action. There are other important aspects both upstream (e.g. synthesis or CBG binding) and downstream (e.g. metabolism or receptor availability) of glucocorticoid concentrations, that could potentially modulate corticosterone effects, in each component of the maternal-placental-foetal triad. Therefore, a small observed difference may have a large impact when it is amplified by other changes, but at the same time, care has to be taken to not overly interpret the biological significance of a statistically significant difference, as compensatory actions can occur in another aspect of the system. It would therefore be prudent to also consider as many other types of evidence as possible, both within the HPA axis and outside of it, as maternal stress activates not just neuroendocrine systems, but also autonomic and immune systems. Larger multi-analyte approaches like the LC-MS method detailed

here, is a good starting point to carry out these investigations. It is also imperative to put each experiment into context and take gestational stage, time of stressor etc into consideration, and not to make comparisons between studies at face value.

Lastly, the fact that the placenta undergoes adaptations following maternal stress support mounting evidence that it is involved in the foetal programming of chronic diseases (reviewed in (Sferruzzi-Perri and Camm, 2016)). Nonetheless, how the placenta regulates the communication between mother and foetus is not straightforward and remains poorly understood. More research focussing on the placenta needs to be carried out, and it remains to be determined which other signals, apart from neuroendocrine signals investigated here, may be contributing to this complex communication at the maternal-foetal interface.

Chapter 6: Role of oxidative stress in the transmission of stress signals from mother to foetus

6.1 INTRODUCTION

In chapter 5, the role of steroids in the transmission of stress signals from the mother to the foetus was explored. It was concluded that although corticosterone and neuroactive steroid concentrations were increased following maternal stress in the maternal component, they were unlikely to participate directly in the transmission of the stress signals. The complexity of the communication between the mother and foetus was also acknowledged, and the results begin to unravel the dynamic and central role the placenta may play in modulating the transmission of the stress signals during pregnancy.

Thus far, the thesis has been focussed on analysing the role of neuroendocrine factors (e.g. corticosterone and neuroactive steroids) in mediating the outcomes of prenatal stress. However, as proposed in the concluding remarks of the previous chapter, maternal stress not only activates neuroendocrine systems, but also immune, metabolic and autonomic systems, and there is likely to be considerable cross-talk between these systems. Additionally, mechanisms of prenatal stress span several levels, and changes at a systems level are likely to also lead to changes at a molecular and cellular level. This prompted the exploration of other biochemical mechanisms outside of the HPA axis in attempting to elucidate the mechanisms of prenatal programming. Several questions start to arise, such as – does stress on an organism level also lead to stress at a cellular level? Could targeting the negative effects stress has at the cellular level be one of the ways in which one can modify the outcomes of prenatal stress in a systemic and organism level?

In the present study, it was hypothesised that stress experienced by the pregnant dam also results in stress at the cellular level, manifesting as increased oxidative stress in all three components of the maternal-placental-foetal triad. Using an antioxidant administered to the mother, which serves both as an experimental manipulation and a possible therapeutic invention, the aim here was to determine the role oxidative stress may play in mediating the effects of prenatal stress, and whether antioxidant therapy to the mother could be a viable intervention for prenatal stress-related pathologies. The study also attempts to dissect the role the placenta may play in the direct transmission of stress signals from the mother to the foetus.

6.1.1 Oxidative stress during pregnancy

Oxidative phosphorylation is the cellular process through which energy, in the form of ATP, is generated from oxygen via a series of reactions in the mitochondrial electron transport chain. While this process is essential for energy production, it also produces reactive oxygen species (ROS), which includes free radicals which are highly reactive due to the presence of unpaired electrons (e.g. superoxide anion), or their non-radical intermediates (e.g. hydrogen peroxide) which still exhibit high reactivity. Whilst ROS are generally regarded as harmful by-products of energy metabolism, in moderate concentrations, they are in fact important regulatory mediators of signalling processes and participate in various cellular functions, such as the regulation of redox-sensitive transcription factors and protein kinases (Droge, 2002). However, in high concentrations, ROS may react with cellular components indiscriminately, leading to lipid peroxidation, DNA/protein oxidation and eventual cellular damage and apoptosis. As such, there exists endogenous antioxidant defences to scavenge ROS, and these protective defences may be enzymatic (e.g. superoxide dismutase (SOD), catalase, glutathione peroxidase) or non-enzymatic (e.g. vitamin C or E) in nature. Therefore, it is only when this balance between ROS and antioxidant defences are perturbed that harmful effects of ROS may manifest. Oxidative stress is therefore defined as the imbalance between a pro-oxidant and an antioxidant state, favouring the pro-oxidant state, which can lead to negative consequences or pathological conditions (Burton and Jauniaux, 2011).

Incidentally, normal pregnancy in itself represents a state of constant mild oxidative stress, due to the higher metabolic demands of growth, especially in the feto-placental unit (Myatt and Cui, 2004). The placenta contributes significantly to superoxide anion production during pregnancy, but at the same time, all the major antioxidant defence systems including catalase, glutathione peroxidase and vitamins C and E are also present in the placenta (Myatt and Cui, 2004). In the mother, a systemic inflammatory response also occurs during pregnancy, resulting in the constant generation of large amounts of ROS in other maternal tissues (Burton and Jauniaux, 2011). Nonetheless, this state of mild oxidative stress seems to be required for the normal progression of pregnancy and normal placental function. During early pregnancy for instance, mild oxidative stress is necessary for normal placentation to occur (Nezu et al., 2017). In the foetal compartment, a certain amount of oxidative stress is also required for normal organogenesis and foetal

growth (Dennery, 2010). Thus, the pro-oxidant to antioxidant ratio needs to be tightly controlled, in the maternal, placental and foetal compartments, to ensure normal progression of the pregnancy. Given how delicate the balance is and how many of these processes involving oxidative stress are inter-linked, any slight perturbation in the balance (e.g. from environmental insults or stress) could set off a cascade of feed-forward loops that can result in deleterious outcomes (Burton and Jauniaux, 2011). Therefore, as much as oxidative stress is a characteristic of normal pregnancies, it is also recognised to play a role in the pathophysiology of pregnancy complications and undesirable offspring outcomes.

6.1.2 Increased oxidative stress in compromised pregnancies

Oxidative stress status in the mother:

In compromised pregnancies (e.g. preeclampsia, gestational diabetes, preterm birth and IUGR) mothers usually exhibit oxidative stress levels that exceed what is considered normal (Cuffe et al., 2017a). In humans, maternal oxidative stress status are easily modifiable by lifestyle factors such as smoking, alcohol intake and diet (Block et al., 2002). Maternal psychosocial factors also play a role, and socioeconomic disadvantage, which is presumably linked to higher psychological stress, is associated with higher oxidative stress levels, detected in urine samples (Eick et al., 2018). Moreover, activation of the HPA axis following various stressors is also associated with increased cellular oxidative stress (Spiers et al., 2014). Depending on the severity of the insults and the extent of the perturbations, maternal oxidative stress can result in slight changes in placental function that do not endanger the pregnancy, or it can lead to severe pregnancy complications.

Clinical studies have shown that the pathological outcomes of maternal oxidative stress differ according to the trimester: oxidative stress during the first trimester is associated with miscarriage, while oxidative stress in the second or third trimester are linked to preeclampsia, gestational diabetes and preterm birth, which can have grave consequences for the foetus (Sultana et al., 2017). Similarly, in rodent studies, greater maternal oxidative stress levels have been observed in experimental models of preeclampsia (Beausejour et al., 2007), gestational diabetes (de Souza et al., 2010) and maternal obesity (Bouanane et al., 2009).

As oxidative stress is often involved in every stage of the pregnancy compromise, changes in maternal oxidative stress levels can therefore be considered (i) a consequence of insults during pregnancy, (ii) a cause of pregnancy complications, and (iii) in general, also a biomarker for compromised pregnancies (Figure 6.1).

Oxidative stress status in the placenta:

Although oxidative stress indices have more commonly been determined from maternal plasma or urine, placenta oxidative stress often lie at the centre of these compromised pregnancies (Burton et al., 2009, Burton et al., 2016, Schoots et al., 2018). Oxidative stress can affect placental function through altering processes involved in trophoblast differentiation, proliferation and apoptosis, or through affecting placental angiogenesis and vascular reactivity and the remodelling of placental blood supply (Myatt and Cui, 2004). Secretory patterns of growth factors and cytokines may also change, for instance in the case of diabetes (Desoye and Hauguel-de Mouzon, 2007) or hypoxia (Malek et al., 2001). These changes in turn all impact foetal growth, which can then lead to compromised foetal outcomes via prenatal programming (Thompson and Al-Hasan, 2012, Burton et al., 2016). For instance, oxidative stress during pregnancy has been implicated in an increased cardiovascular risk for the offspring during adulthood, in both human studies and in experimental animal models (Rodriguez-Rodriguez et al., 2018).

Oxidative stress in the foetus:

Given that redox processes are also important in foetal growth, it is likely that the above perturbations can lead to changes in oxidative stress status in the foetus as well (Dennery, 2010). For instance, maternal exposure to environmental pro-oxidants also causes increased oxidative stress in the foetal brain 24 hr later, consequently resulting in microglia activation and inflammation which can lead to neurodevelopmental anomalies (Akhtar et al., 2017).

As maternal oxidative stress is intracellular, the transmission of this oxidative stress signal from mother to foetus will be indirect, possibly through above-mentioned changes in the placenta, which then subsequently cause foetal oxidative stress. Impacts of these oxidative changes in the foetal brain can still be observed in the brains of adult offspring, for instance, in the form of mitochondrial damage in the hippocampus (Song et al., 2009). Additionally, given that oxidative changes can

underlie many neuropsychiatric disorders (Salim, 2014) and metabolic disorders (Roberts and Sindhu, 2009), it is therefore not a surprise that oxidative stress is involved in every stage of developmentally-linked disease, from foetal programming to the manifestation of the disease at adulthood (Fig 6.1).

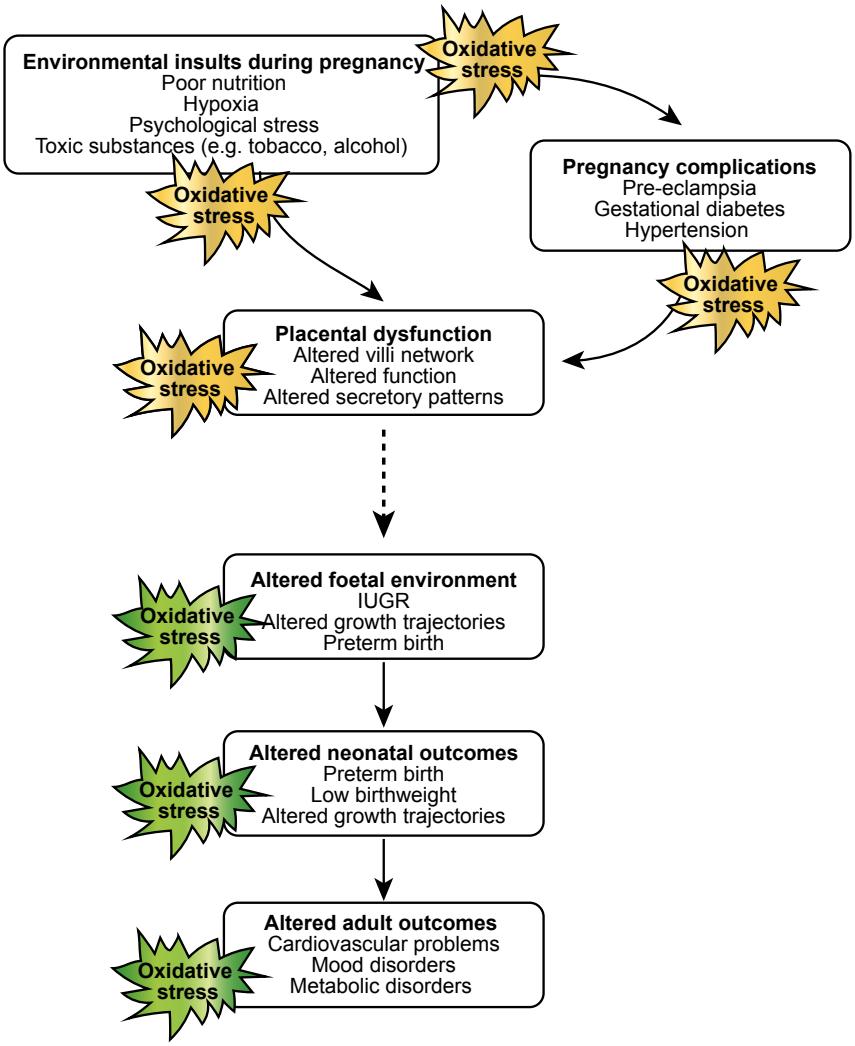


Figure 6.1: The involvement of oxidative stress in all stages of compromised pregnancies, thereby causing foetal programming and poor offspring outcomes. Maternal and placental oxidative stress is coloured in yellow while oxidative stress experienced by the offspring is shown in green. As maternal oxidative stress is intracellular, the transmission of this oxidative stress signal from mother to foetal tissues will be indirect.

6.1.3 Mitochondrial dysfunction in compromised pregnancies

At an organism level, the placenta is responsible for the large amounts of ROS produced, but at a cellular level, the mitochondria are the main site of ROS production. Mitochondria are the energy powerhouse of the cell and is involved in energy production via oxidative phosphorylation at the electron transport chain, rendering it very susceptible to ROS-mediated damage and the impacts of cellular stress (Javadov and Kuznetsov, 2013). Mitochondria are multifunctional organelles, apart from their central role in meeting cellular energy demands, they also regulate other cellular functions like apoptosis (Holland et al., 2017). Altered mitochondrial function can then in turn impact placental function, and influence processes such as glucose transport and even steroidogenesis, ultimately playing a role in the foetal programming of brain functions (Hoffmann and Spengler, 2018). Mitochondrial behaviour is also extremely dynamic, and they can alter their shape via fusion or fission, and also increase or decrease in numbers in response to stressors (Youle and van der Bliek, 2012). In addition, once slight damage to the mitochondrial membrane or proteins occur, it may also drive a positive feedback loop and feed-forward mechanism where it triggers the failure of an entire network of mitochondria (Aon et al., 2006).

The mitochondria have also recently been suggested to play an integral role in modulating the effects of psychological stress. Through a systematic review of both animal and human studies, Picard and McEwen have gathered evidence that acute and chronic stressors influence mitochondrial function, mostly in an adverse manner, especially in the brain (Picard and McEwen, 2018b). They went on further to introduce the concept of mitochondrial allostatic load, and proposed that the mitochondria represent a major intersection point between endocrine and immune systems that together can affect the outcome of psychological stress (Picard and McEwen, 2018a). Therefore, given that allostatic load at an organism and system level are both increased during gestational stress and early life stress respectively (section 1.2.2), it is likely that on a cellular level, alterations in mitochondria allostatic load could also occur in these conditions.

Mitochondria dysfunction also seem to be related to anxiety-related social disorders, which could be pertinent for the model of social stress used in this study. Anxious rats that are prone to become subordinate during a social encounter exhibit reduced

mitochondrial complex I and II protein, have decreased ATP production and increased ROS production in the nucleus accumbens (Hollis et al., 2015). In terms of the HPA axis, mitochondria can also change their function and morphology in response to glucocorticoids (Hoffmann and Spengler, 2018), and an increase in protein carbonylation and mitochondrial dysfunction has also been observed with increased glucocorticoids in *in vitro* studies using a rat cell line (Tang et al., 2013). As such, the links between maternal psychological stress and foetal programming could perhaps be narrowed down to the dysfunction of mitochondria at a cellular level.

6.1.4 Antioxidant treatments during pregnancy

Theoretically, given the central role of oxidative stress in pregnancy complications, antenatal antioxidant therapy should reverse these processes associated with oxidative damage and negative gestational outcomes. Therefore, there has been tremendous interest in the use of antioxidants to protect the foetus against the harmful effects of oxidative stress in conditions such as preeclampsia (Miller et al., 2012). However, as much as *in vitro* and preclinical studies have showed potential, antioxidant therapy in human clinical studies in recent decades have not produced promising results (Duhig et al., 2016). Extensive clinical studies have been carried out on the use of vitamin C and E during pregnancy to prevent complications associated with preeclampsia, yet most studies concluded that vitamin C and E have no effect in preventing preeclampsia or improving foetal outcomes (Poston et al., 2006, Rumbold et al., 2006, Polyzos et al., 2007). As ROS play important roles endogenously, the timing and dosage of antioxidant administration, the type of antioxidant given, and how it is given, could all be factors that affect its efficacy. The fact that these clinical trials have yielded disappointing results perhaps also suggests that these protective agents were not being localised to where they need to be (for instance, the mitochondria, or the placenta) in order to counter the oxidative damage (Smith and Murphy, 2011).

In recent years, several mitochondria-targeted antioxidants have been developed (Smith and Murphy, 2011). Mitochondria-targeted antioxidants are chemically tagged versions of natural antioxidants such as vitamin E and coenzyme Q, which modifies their physical properties to allow for their concentration in the mitochondria. These drugs were found to be stable, well-tolerated and efficacious in rodent studies

when ingested through the drinking water, and they protected the mitochondria from oxidative damage far more effectively than untargeted antioxidants, indicating their improved efficacy (Smith et al., 2003). In rats, mitochondrial-targeted antioxidants are also effective in countering oxidative stress in various experimental models of disease, such as cardiac injury, Parkinson's disease, and Alzheimer's disease (Smith and Murphy, 2011). In rat hypoxic pregnancies, orally administered mitoquinone (MitoQ) treatment in pregnant rats increases placental perfusion and prevents the activation of mitochondrial stress, suggesting that mitochondria-targeted antioxidants may indeed be beneficial in preventing pregnancy complications (Nuzzo et al., 2018).

Apart from targeting the mitochondria on a cellular level, in order for an antioxidant to be able to successfully exert its effects, on an organism/system level, it also has to reach the target tissues (i.e. the placenta) in sufficient concentrations, but also not cause any adverse or unintended off-target effects on the foetus. There is a severe lack of progress in the past decades in developing interventions that can target placental dysfunction, due to the difficulties in preventing the treatment in the mother from affecting the foetus (Sibley, 2017). In recent years however, there has been a renewed interest in the development of strategies to deliver drugs and therapeutic agents directly to the placenta, for instance, with the use of drug delivery systems with placental-homing peptides that can deliver drugs specifically to placental trophoblast cells, without affecting the foetus (King et al., 2016).

6.1.5 MitoQ-NP

In this study, MitoQ-NP, which consists of the antioxidant mitoquinone (MitoQ) loaded in a nanoparticle delivery system (NP), was used as the antioxidant to counter oxidative stress. MitoQ is made up of coenzyme Q covalently attached to a lipophilic triphenylphosphonium (TPP) cation. Coenzyme Q is an important component of the electron transport chain, and is a major antioxidant, where it exerts protective effects on lipids, protein and DNA (Bentinger et al., 2007). When administered to pregnant women, coenzyme Q was shown to reduce the risk of preeclampsia (Teran et al., 2009). The lipophilic TPP cation of MitoQ allows for the compound to be concentrated a thousand fold within the mitochondria as compared to other cellular compartments (due to the presence of the large mitochondrial

membrane potential), thereby enhancing the beneficial effects of coenzyme Q in the mitochondria (Murphy and Smith, 2007).

Nanoparticles are defined as particles ranging from 1 to 1000 nm in size, and have a wide variety of uses. Nanoparticles as drug delivery systems can exist as liposomes, micelles or a matrix, where drugs can be adsorbed, dissolved, conjugated or encapsulated (Singh and Lillard, 2009). Nanoparticles are advantageous in that they aid stability of the drug when it is transported in the bloodstream, allowing for lower doses of the drug to be used. Depending on the material of the nanoparticle, the use of biodegradable materials can also allow sustained release of the drug over a long period of time (Singh and Lillard, 2009). There is great potential for the manipulation of physical and chemical properties of the nanoparticles, such as size and surface chemistry, which allows the optimal delivery of the drug to the target tissues. It can therefore be potentially useful in delivering drugs to the placenta without affecting the foetus. Transplacental passage of nanoparticles are regulated by their size (Refuerzo et al., 2017) and in general, nanoparticles that are of size 100nm will not cross the trophoblast layer into the foetus (Menezes et al., 2011). The nanoparticle used in this study has a size of 180 nm, and contains an outer hydrophilic shell made up of a naturally occurring poly-amino acid poly(γ-glutamic acid) (γ-PGA), which aids the stabilisation of the nanoparticle in the bloodstream. It has an inner hydrophobic core made up of L-phenylalanine ethylester (Phe), allowing for the adsorbtion of the hydrophobic drug (Kim et al., 2009).

In an *in vitro* model of a bilayered trophoblast barrier using a placental trophoblast-derived choriocarcinoma cell line, it was previously shown that these γ-PGA-Phe nanoparticles accumulate on the top layer of the trophoblasts and do not pass through it (Sood et al., 2011). In the case of *in vivo* administration of MitoQ-NP, the drug does not cross the placenta barrier and were not found in the foetal liver nor in the foetal brain (Phillips et al., 2017). However, as these NPs do not have a placental-homing component (King et al., 2016), apart from being present in large amounts in the placental trophoblasts, they were found to also located in the maternal brain and liver (Phillips et al., 2017).

6.1.6 Effects on MitoQ-NP on offspring outcomes in compromised pregnancies

MitoQ-NP has been previously used in two separate *in vivo* rat studies and was found to be efficacious in preventing the increase of ROS in maternal brain, maternal liver and placenta following maternal hypoxia. The negative effects on the adult offspring were also rescued, indicating that increased oxidative stress is likely to be a mechanism involved in foetal programming associated with maternal hypoxic stress. In these studies, pregnant rats were intravenously injected with MitoQ-NP on GD15 and were then immediately exposed to hypoxic conditions for 6 (Phillips et al., 2017) or 7 days (Aljunaidy et al., 2018). In both cases, when pregnant rats were killed at the end of the hypoxic stress procedure, increased ROS production in the placenta was observed, and MitoQ-NP was effective in preventing this increase. Fluorescent labelling also showed that the NPs are sequestered in the trophoblast layer of the placenta and did not cross over to the foetus (Phillips et al., 2017).

In the first study (Phillips et al., 2017), maternal MitoQ-NP treatment prevented the lower birthweight in offspring with dams that underwent hypoxia. When neuroanatomical markers were examined in PND30 offspring, it was noted that maternal Mito-NP could normalise changes in the offspring brain associated with maternal hypoxia, such as decreased dendritic length and increased tyrosine hydroxylase positive (TH+, a marker for dopaminergic neurons) cell process length in the thalamic reticular neurones. The decrease in the number of parvalbumin (PV+, a marker for GABAergic neurones) positive cells in the offspring brain following hypoxia was also rescued with maternal MitoQ-NP, indicating an effect of maternal MitoQ-NP treatment on offspring GABAergic neurone development. When transcriptome analysis was carried out on the foetal brains, it was observed that maternal MitoQ-NP could partially normalise some of the 510 genes that were differentially regulated compared with the non-hypoxic controls.

In the later study (Aljunaidy et al., 2018), whether maternal MitoQ-NP treatment could prevent programming of cardiovascular dysfunction was subsequently explored. Male and female rat offspring were assessed during adulthood (at 7 and 13 months) for cardiac and vascular function. At 7 months, while male and female offspring presented the same cardiac diastolic dysfunction, maternal MitoQ-NP treatment rescued cardiac dysfunction only in the female offspring, but not in the

males. At 13 months, vascular sensitivity to vasoconstrictors were analysed and there were sex- and age-dependent effects of hypoxia and maternal MitoQ-NP treatment. This study showed that changes induced by stressors and maternal antioxidant treatment during gestation can have significant impacts on the offspring phenotype during adulthood and more than 1 year after birth.

As the maternal stressor used in these two previous studies were hypoxia, it is not known if the same observations of increased placental ROS production, and subsequent rescue with MitoQ-NP treatment, will be observed if other stress paradigms are used. Here, using the same treatment regimen (where one dose of MitoQ-NP was given intravenously to the pregnant dam, prior to the induction of the stress), it was investigated if MitoQ-NP treatment could bring about similar beneficial effects in the case of maternal social stress.

Previously, maternal MitoQ-NP treatment was shown to rescue neuroanatomical defects such as decreased dendritic length and GluN1 expression in the cortex following hypoxic stress (Phillips et al., 2017), indicating that these outcomes are under the control of maternal mitochondrial oxidative stress during pregnancy, and are sensitive to perturbations *in utero*. It is therefore possible that these neuroanatomical deficits may also be observed in the social stress model, in limbic regions such as the hippocampus and the amygdala. In addition to changes in dendritic length and GluN1 expression, it is possible for alterations to occur in $GABA_A$ and $GABA_B$ receptor subunit expression, as GABA receptor expression patterns are also sensitive to stress-related changes in early life (Skilbeck et al., 2010). They also play an important role in the modulation of anxiety behaviour, responses to stress, and mediate the action of neuroactive steroids during adulthood, all of which may be impaired in PNS offspring (Gunn et al., 2011). Changes in $GABA_A$ receptor subunit composition have been shown to occur following chronic stress (Locci and Pinna, 2017), and can underlie the development of other stress-related neuropsychiatric diseases in adulthood (Jie et al., 2018).

In this study, GABA and NMDA receptor expression patterns will be analysed alongside several behavioural tests, to determine if MitoQ-NP is also able to rescue offspring deficits associated with prenatal social stress exposure. Two $GABA_A$ subunits $GABA_{A\alpha1}$ (*Gabra1*) and $GABA_{A\alpha2}$ (*Gabra2*), the $GABA_{B1}$ (*Gabbr1*) subunit, and the GluN1 subunit of the NMDA receptor (*Grin1*) will be investigated. The

structure of the $GABA_A$ and NMDA receptor, especially with respect to their regulation by neuroactive steroids, was introduced in Chapter 1. The $GABA_B$ receptor, on the other hand, are not known to be targets of steroids (Cryan and Kaupmann, 2005). The $GABA_B$ receptor is heterodimeric, made up of the B1 and B2 subunits. Each of the subunits are G-protein coupled receptors, and upon binding of GABA, exert downstream effects by phosphorylation-mediated mechanisms (Enna, 2007).

6.1.7 How is the oxidative stress signal transmitted to the foetus?

In as much as oxidative stress is observed to be increased in the placenta following insults, and maternal MitoQ-NP treatment is able to prevent this increase, oxidative stress is ultimately an intracellular phenomenon, and there needs to be a way in which this is communicated to the foetus, to result in the observed detrimental effects. Oxidative stress may cause tissue damage, resulting in the changes in the fine structure of the placenta, and therefore the altered transport of nutrients. However, in the case of hypoxia, the placenta did not seem physically or structurally altered, as there were no changes in the size of labyrinth zone or maternal decidua, nor in the foetal capillary network in the labyrinth zone following the stressor or MitoQ-NP treatment (Phillips et al., 2017). It may be that placenta structure is less amenable to changes during late pregnancy, as the process of placentation occurs in the earlier stages of pregnancy. Since increased oxidative stress did not result in structural changes in these studies, it was proposed that the secretory functions of the placenta could be impaired.

By using human placenta explants or a model placenta barrier maintained in culture media, it was first shown that the placenta can indeed respond to hypoxic stress *in vitro* by secreting damaging 'factors' into the culture media (Curtis et al., 2014). When the conditioned culture media containing these damaging "placental secretions" were applied to embryonic cortical neurones *in vitro*, these neurones exhibited reduced synaptic activity, dendritic length, branching complexity and spine density. When the same conditioned culture media containing these "placenta secretions" were injected into a developing brain of a PND4 rat, there was a decreased density of PV+ neurones in the developing rat's cortex, hippocampus and reticular nucleus, a marker of GABA producing neurones, suggesting that these

damaging factors have the propensity to directly affect brain development, especially the development of GABAergic circuits.

A similar investigation was carried out in Phillips et al. (2017), and the culture media in this instance was conditioned by placenta from hypoxic pregnancies (the pregnant dam was exposed to 6 days of hypoxia as described in section 6.1.5). The culture media (also termed 'conditioned media') was then applied to embryonic cortical neurones in a similar manner, and there were decreases observed in dendritic length, process lengths of tyrosine hydroxylase (*Th*; a marker for dopaminergic neurones) positive neurones (TH+) and a decrease in GluN1 receptor expression. Some of these observations could be recapitulated in certain brain regions of adult offspring, described in section 6.1.5, suggesting that the changes observed in the adult brain could be due to the direct effect of the damaging factors produced by the hypoxic placenta on neurones. Additionally, when foetal plasma was collected and also applied to embryonic cortical neurones, similar *in vitro* patterns of alteration as that of the "conditioned media" were observed, suggesting that the damaging factors secreted by the placenta *ex vivo* was possibly being secreted into the foetal circulation. It was therefore hypothesised that these damaging factors affected the foetal brain *in utero* and neuroanatomical changes persisted into adulthood, and therefore plays a direct and crucial role in foetal programming.

In this study, ithe same procedure was performed, where placental-conditioned media and foetal plasma from stressed and MitoQ-NP treated animals was collected and then applied to neuronal cultures *in vitro*. This could provide clues on whether "placental secretions", associated with placental oxidative stress, could be the factors that are also directly involved in the transmission of social stress signals from mother to foetus.

6.1.8 Aims of this chapter

This study aims to use MitoQ-NP administered to the pregnant mother as an experimental manipulation to establish if, and how, oxidative stress may play a role in mediating the effects of prenatal social stress. At the same time, the study aims to also determine whether this mitochondrial-targeted and nanoparticle-delivered antioxidant therapy administered to the mother could be a viable intervention for prenatal stress-related disorders in the offspring.

This study spans two developmental time points, where two experiments on two separate cohorts of rats were carried out. Pregnant rats in cohort 1 were killed during GD20 to investigate the direct effects of the MitoQ-NP treatment during gestation on the maternal, placental and foetal compartments. In cohort 2, the offspring were tested during adulthood to ascertain the effects of maternal MitoQ-NP treatment on behavioural and neuroanatomical phenotypes (Figure 6.3).

It was first investigated whether maternal and/or foetal ROS levels were altered by gestational social stress, which would point to the involvement of oxidative stress in the transmission of stress signals and/or foetal programming. It is hypothesised that maternal social stress, like maternal hypoxia exposure, can increase the oxidative stress status of the mother and placenta.

Then, it was investigated if maternal MitoQ-NP treatment could rescue the behavioural and/or biochemical deficits observed in the adult offspring when they have been prenatally stressed using the gestational social stress model. It is hypothesised that maternal MitoQ-NP treatment, which prevents the increase in maternal oxidative stress, may rescue the deficits seen in the adult PNS offspring.

Next, assuming that oxidative stress is indeed involved and MitoQ-NP can indeed normalise aberrant phenotypes, *how* the oxidative stress signal is transmitted from the mother and dam is then explored. The focus is placed on the placenta, where a similar *ex vivo* experiment was carried out (as in section 6.1.7), to determine if there were such “damaging factors” in the placentae from socially stressed dams. It is hypothesised that deficits observed in PNS offspring in adulthood can arise from prenatal programming at the foetal stage, as a result of “damaging factors” secreted by the placenta into the foetal circulation.

Finally, it was also determined whether MitoQ-NP administration could potentially affect the pregnant dam in any other way or if there are any other knock-on effects, thus maternal plasma steroid concentrations and placenta 11β-HSD2 positive cell counts were also quantified. It is hypothesised that there is likely to be considerable cross talk between cellular stress and neuroendocrine responses/glucocorticoid regulation following stress.

Specific questions, summarised in Fig 6.2, are as follows:

1. Is oxidative stress status altered with gestational social stress? Can it be prevented by MitoQ-NP administration? (cohort 1)
    - Maternal oxidative stress status: Are ROS levels increased in the placenta, maternal brain and liver?
    - Foetal oxidative stress status: Are ROS levels increased in the foetal brain and liver?
    - If so, can maternal MitoQ-NP treatment prevent the increased ROS levels in the mother placenta and the foetus?

2. Can the prevention of oxidative stress (via maternal MitoQ-NP treatment) rescue the adverse phenotypes associated with prenatal social stress in adult offspring? (cohort 2)
    - Can maternal MitoQ-NP treatment rescue the anxious behaviour in adult male offspring? (Light-dark box and elevated plus maze)
    - Is there a depressive-like phenotype in the offspring, and does maternal MitoQ-NP treatment alter it? (Sucrose preference test and forced swim test)
    - Can maternal MitoQ-NP treatment rescue the social memory deficits in adult female offspring? (Social olfactory memory test)
    - Can maternal MitoQ-NP treatment normalise HPA axis hyperactivity observed in offspring following restraint stress?
    - Can maternal MitoQ-NP treatment alter biochemical changes in the brain of adult offspring?
        - CRH mRNA expression in central amygdala (linked to anxious behaviour)
        - In the basolateral amygdala and hippocampus (CA1, CA2 and CA3):
            - GluN1 protein expression
            - GABA receptor subunits ($GABA_{A\alpha1}$, $GABA_{A\alpha2}$ and $GABA_{B1}$) protein expression
            - Parvalbumin positive cell count
            - Dendritic length of neurones

3. How is the oxidative stress signal being transmitted to the offspring and what is the role of the placenta? (cohort 1)

- Is the placenta secreting damaging factors that are causing changes to foetal cortical neurones? Does MitoQ-NP treatment prevent the effects of these damaging factors?
- Are these damaging factors present in the foetal circulation? Does MitoQ-NP treatment prevent the effects of these damaging factors?

4. Are neuroendocrine outputs in the maternal component altered? (Batch 1)
    - Does maternal MitoQ-NP impact the HPA axis response of the mother?
    - Does maternal MitoQ-NP treatment alter neuroactive steroid production in the mother?
    - Can MitoQ-NP alter other functions of the placenta (e.g. 11β-HSD2)?

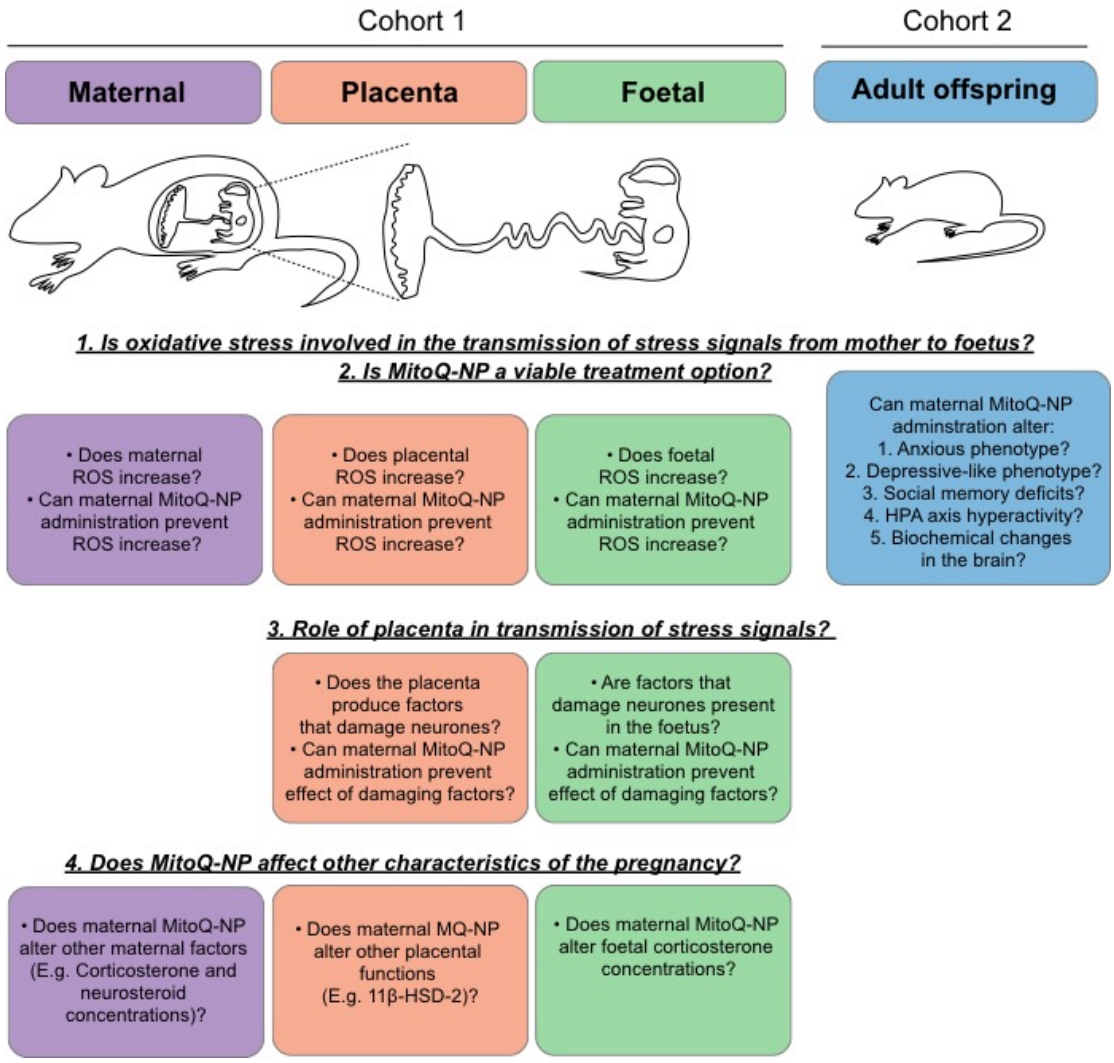


Figure 6.2: Summary of study aims and specific questions

6.2 METHODS

6.2.1 Preparation of MitoQ-NP drug

MitoQ-NP was prepared in the University of Bristol by Dr Tom J Phillips, in the same manner as previously described (Phillips et al., 2017). Mitoquinone (MitoQ) was a kind gift from the laboratory of Prof Mike Murphy, MRC Mitochondrial Biology Unit, University of Cambridge (Kelso et al., 2001, James et al., 2005). Various patents for MitoQ have been held by Prof Mike Murphy and the company Antipodean Pharmaceuticals. The γ-PGA-Phe nanoparticles (NPs) used in this study were a kind gift from the laboratory of Prof Mitsuru Akashi, Osaka University (Kim et al., 2009). Briefly, equal volumes of γ-PGA-Phe NPs (10 mg/mL) and MitoQ (2 mg/mL) were mixed in 0.2 M NaCl, and incubated at 4°C for 12 hr, resulting in MitoQ being adsorbed by hydrophobic and electrostatic interaction to these γ-PGA-Phe NPs. The mixture containing MitoQ-NP was then centrifuged, washed and resuspended in PBS, into a concentration of 125μM MitoQ-NP for injection. MitoQ-NP was transported from University of Bristol to the Roslin Institute in a chilled shipment with ice packs. Application of a patent for the use of MitoQ-NP as a drug to target compromised pregnancies by the University of Bristol is ongoing (Case et al., 2016).

6.2.2 Animals, drug administration and social stress

Two batches of experiments were carried out in this study. Cohort 1 was focussed on the pregnant dam, placenta and foetus during GD20, while cohort 2 focussed on the offspring during adulthood. Gestational social stress and drug administration were carried out in the same manner for both cohorts.

The generation of pregnant rats and social stress procedure were carried out as described in section 2.2. Briefly, female Sprague Dawley rats (n=7-8 each group) were mated with a male and the morning in which the vaginal plug was found was designated as GD1. Pregnant rats were left undisturbed and group housed until GD16. On GD16, rats were weighed and 4% (w/w) lidocaine cream was applied topically to the tail before they were placed in a warm chamber for 5 min for tail vein dilation. Rats were gently restrained in a towel and the injection was performed by experienced animal technicians from the Roslin Institute Biological Resource Facility. 125μM MitoQ-NP was injected intravenously into the tail vein at a volume of 0.34 ml/kg, and 0.9% sterile saline was injected as the vehicle. The volume was calculated to ensure at least 0.5μM of MitoQ-NP in the maternal circulation upon

injection, a dose which was shown to be effective in counteracting oxidative stress in previous *in vivo* and *in vitro* studies (Phillips et al., 2017). Approximately 1 hr after the injection, rats were exposed to 10 min social stress, using the modified resident-intruder paradigm as previously described (Chapter 2). Social stress was repeated on GD17 to GD20 (i.e. on 5 consecutive days). Non-stressed controls were returned to individual IVCs after the injection. This experimental set-up generated four treatment groups: Control/Vehicle, Stress/Vehicle, Control/MitoQ-NP and Stress/MitoQ-NP.

For cohort 1, after the final stressor on GD20, the pregnant rats were killed by conscious decapitation and maternal, placental, and foetal tissues were collected (section 6.2.5.1).

For cohort 2, after the final stressor, pregnant rats were returned to their home cages and were left undisturbed until parturition. Offspring stayed with the dams throughout lactation until postnatal day (PND) 23, where they were weaned and separated into cages according to sex. Offspring were group housed with their littermates, so that each cage had approximately 5-7 rats. Various procedures were carried out on the offspring at different ages, listed on Table 6.1. One rat of each sex was used from each litter to minimise cage/litter-related effects.

Cohort	Age	Sex	Procedures	Kill method	Tissues
2-1	PND30	1 male and 1 female per litter	None	Perfusion	Brain: IHC
2-2	PND30	1 male per litter	None	CO_2 overdose/ decapitation	Brain: ISH
2-3	Beginning from 9 weeks old	1 male and 1 female per litter	Anxious/depressive-like behaviour: 1. Light-dark box 2. Elevated plus maze (4 days later) 3. Sucrose Preference Test (4 days later) 4. Forced Swim Test (4 days later) N.B.: Rats were returned to home cage after each test	CO_2 overdose and decapitation (1 week after forced swim test)	Brain: ISH
2-4	12 weeks old	1 male and 1 female per litter	Surgical cannulation 4 days prior to blood sampling	CO_2 overdose	None
2-5	15 weeks old	1-2 females per litter	Social olfactory memory testing	CO_2 overdose	None

Table 6.1: Details of juvenile and adult offspring used for experiments. Each batch of offspring were used for different purposes and were killed at the end of the procedure. IHC: immunohistochemistry, ISH: *In situ* hybridisation.

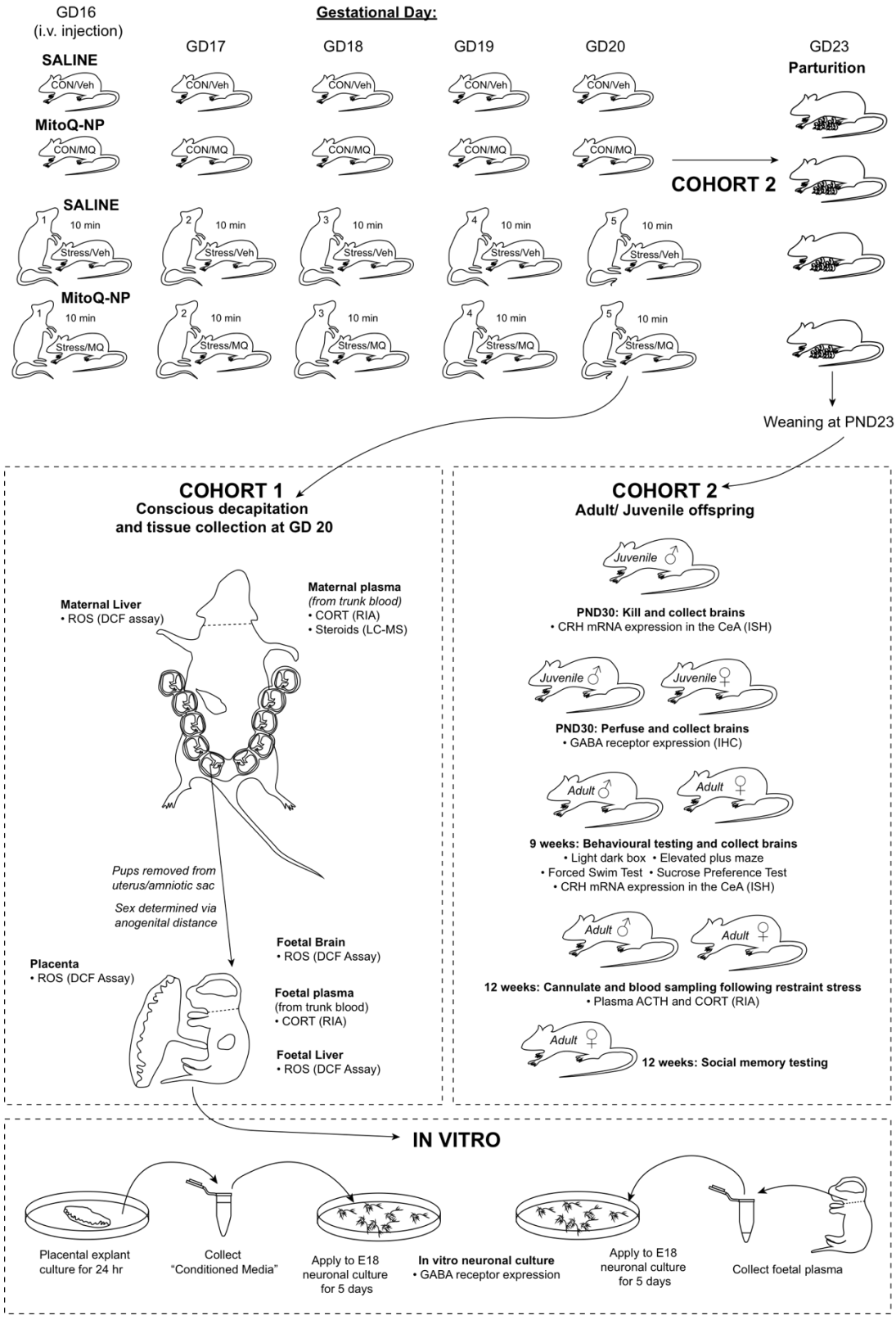


Figure 6.3: Experimental set up. Two cohorts of rats were used in the study.

6.2.3 Assessment of behavioural phenotypes

The light-dark box (LDB), elevated plus maze (EPM), sucrose preference test and forced swim test (FST) were conducted on the same rats to determine anxiety- and depressive-like behaviour. To minimise the effects of behavioural experience on results, experiments were conducted on the rats from the least to the most stress-inducing in the above-mentioned order, with a few days of rest between each test (cohort 2-3, Table 6.1). Apart from the sucrose preference test, where isolated housing was necessary for three days, rats were returned to their home cage and housed with other littermates after each test. Behavioural testing occurred in a separate room and rats were brought to the room immediately before a test in a transport container. Social olfactory memory test (section 6.2.3.3) was carried out on a separate group of female rats (cohort 2-5, Table 6.1).

6.2.3.1 Anxious behaviour

The light-dark box (LDB) (Bourin and Hascoet, 2003) and elevated plus maze (EPM) (Pellow and File, 1986) are two tests that assess anxiety-like behaviours, specifically, the approach-avoidance aspect of anxiety. Behaviour in the test is based on the innate aversion of rats to open and brightly lit spaces, and reflects the conflict between the desire to explore a novel environment and the distress of being in an open and brightly lit area. At ca. 9 weeks of age, rats were first tested on the LDB and were returned to the home cage for four days, before being tested again on the EPM.

***Light-dark box*:** The light-dark box consisted of a transparent Perspex box and a black lidded opaque Perspex box (both 40 x 40 x 40 cm), placed side by side, with an opening (10 x 8 cm) for the rats to freely move between these boxes (Figure 6.4A). Rats were introduced into the dark chamber, and were allowed to explore freely for 5 mins. A decreased amount of time spent in the light chamber or a longer latency to enter indicate anxiety-like behaviour.

Elevated plus maze: The EPM consisted of 2 open arms and 2 closed arms (each 60 cm long and 10 cm wide) forming a plus shape, elevated 60 cm above the ground (Figure 6.4B). The 2 closed arms, which are located opposite each other, have 35 cm high black walls. Rats were introduced to the centre of the maze where the arms crossed, facing one of the open arms, and were allowed to explore the

maze freely for 10 mins. A reduced amount of time spent or lower number of entries in the open arms indicate anxiety-like phenotype.

For both tests, the test arena was back-lit with infrared lighting and the entire duration of the test was recorded and animals tracked with an infrared video camera. The time spent in each zone, latency to enter each zone, number of entries into each zone, and distance travelled were monitored and automatically coded using EthoVision XT software v12 (Noldus, Wageningen, Netherlands). Zones were manually drawn using tools available in the software and a test run was carried out before each cohort to optimise camera and video contrast settings, which ensures the fidelity of the automatic coding. The test arenas were cleaned with 70% ethanol followed by distilled water and dried between each test animal to remove any olfactory cues. Males and females were tested on separate days.

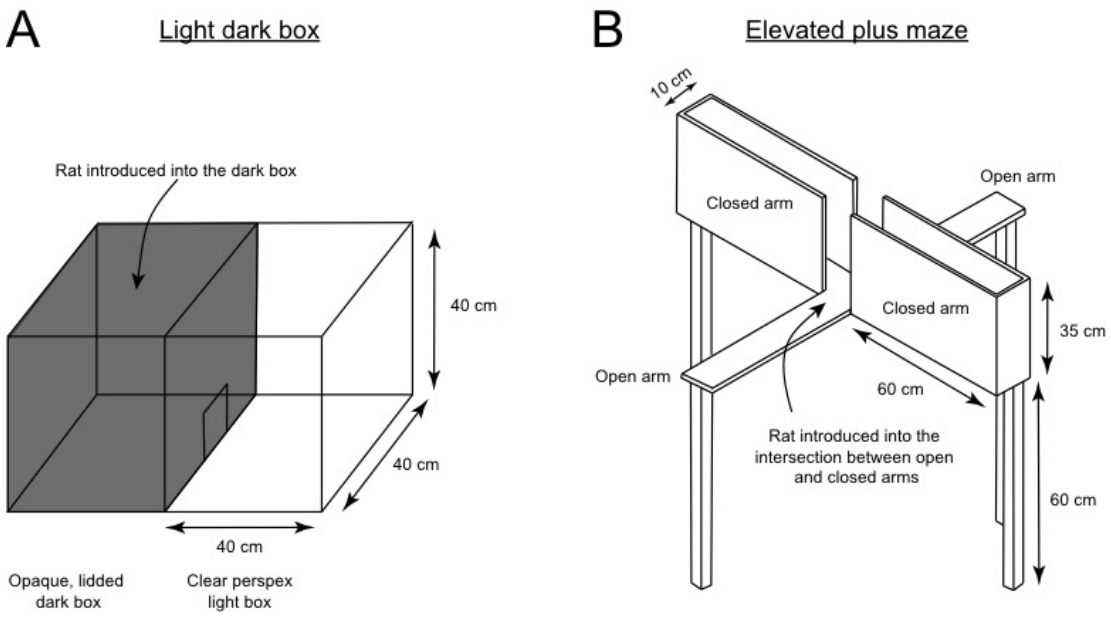


Figure 6.4: Anxious behaviour was characterised with the light-dark box (A) and elevated plus maze (B).

6.2.3.2 Depressive-like phenotype

Two different aspects of a depressive-like phenotype was tested in this study, when offspring were ca. 11 weeks of age. Sucrose preference test was used to assess

anhedonia (Moreau, 2002), while the forced swim test (Porsolt et al., 1978) was used to characterise behavioural despair.

Sucrose preference test: Rats were given the choice to consume either water or 2% sucrose for a 48 hr period. Rats were singly housed in clean IVCs with two bottles per cage one day prior to the first testing day, with two bottles both containing filtered tap water. On the first testing day, one water bottle remained as filtered tap water while the other was substituted for 2% sucrose. After 24 hr, the bottles were weighed and the positions of the water and sucrose bottles were swapped, to reduce any side preferences. The test continued for another 24 hr, after which bottles were weighed again. Preference for sucrose consumed over the 48 hour period was calculated by (sucrose consumption in 48 hr)/(total amount of fluids in 48 hr) x 100%. Preference for sucrose consumed on separate testing days were also calculated by (sucrose consumption in 24 hr)/(total amount of fluids consumed in 24 hr) x 100% to determine if there were any variabilities across testing days.

Forced swim test: Despite its criticisms (de Kloet and Molendijk, 2016, Commons et al., 2017), the forced swim test is widely used as a model for assessing depressive-like behaviour, based on its predictive validity in screening for antidepressants (Lucki, 1997, Slattery and Cryan, 2012), where drug efficacy relates to a decrease in immobility time. The same glass cylinder as in the acute swimming stress procedure in Chapter 4 (diameter, 25 cm; height, 50 cm) was filled with water (22-23^{o}C) to a depth of 30 cm. However, unlike the acute swim stress paradigm, the forced swim test involves two swimming bouts spaced 24 hr apart. On the first day ("pre-test" day), the rats were forced to swim for 10 min in the cylinder, and were gently dried using a towel and returned to the home cage thereafter. This pre-test exposure to the stressor ensures baseline mobility on the next day ("test" day), which allows for differences between treatments to be more easily observed (Slattery and Cryan, 2012). Rats were forced to swim for 5 min on the "test" day, and videos were recorded and coded manually using EthoVision v12, with a pharmacologically validated time-sampling technique (Lucki, 1997). In this sampling method, the 5 minute bout was divided into sixty 5 second bins, and the predominant behaviour in each bin was recorded. Three behaviours were recorded, namely (i) climbing, where there is vertical movement of the forepaws against the walls of the cylinder, (ii) swimming, where there is horizontal or circular movement in the cylinder, with the crossing of quadrants, and (iii) floating, where the rat assumes

a passive position without struggling, making only movements necessary (e.g. gentle paddling using only one foot) to keep its head above the water.

6.2.3.3 Social olfactory memory testing

Social olfactory memory testing was carried out only in the female offspring at ca. 12 weeks of age (cohort 2-5, Table 6.1) as it was previously shown that PNS females, but not males, showed a deficit in social olfactory memory (Grundwald et al., 2016). Wooden beads (25 mm diameter) were impregnated with a social odour by being kept for 5 days in a cage housing unfamiliar female conspecifics. This was based on the observation that urinary scents are among the strongest chemical signals in rodents, encoding complex information that regulate rodent social behaviour (Beynon and Hurst, 2004).

The social olfactory memory test consists of two sessions, an “exposure” phase and a “choice” phase. Before the “exposure” phase, rats were placed in the test arena (i.e. a clean open top cage without bedding) for acclimatisation for 2 hr, with food pellets and water provided. In the “exposure” session, rats were first introduced to a wooden bead (designated as the “familiar” bead) and were allowed to freely interact with it for 4 min. Time spent interacting with the “familiar” beads was recorded to ensure rats investigated the object adequately (>5 s of interaction time). The beads were immediately disposed of after the exposure, and the cages were wiped with 70% ethanol to remove any residual odour. Rats remained in the test arena and the testing room during a 3 hr “consolidation” period. After 3 hours, the “choice” session was carried out where rats were exposed to two beads simultaneously for 4 min, a fresh “familiar” bead (with the same odour as before) and a bead with a “novel” odour. Rats were recorded on video camera and the time spent interacting with each object (which includes sniffing, biting, or holding object) was scored manually on Observer XT (Noldus). Preference score was calculated as time spent investigating “novel” bead divided by the total time spent investigating both beads during the testing session. A total of 8 different scents were used, and “familiar” and “novel” scents were assigned at random for each rat.

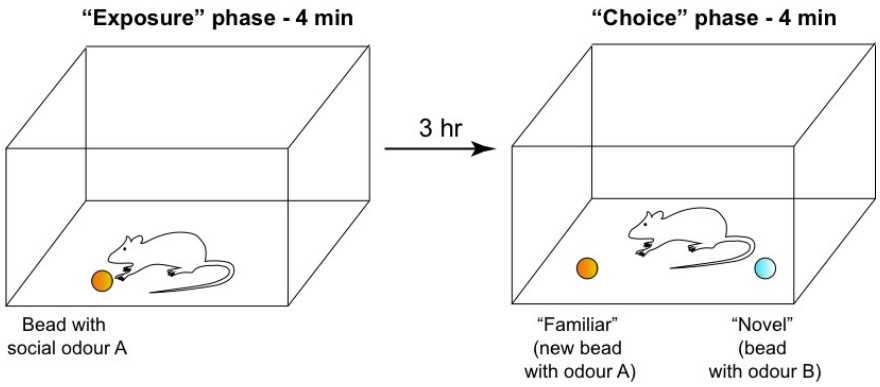


Figure 6.5: Diagrammatic representation of the social olfactory memory test carried out in female rats. Rats were first exposed to a bead with a "familiar" odour, and testing was carried out 3 hr later where rats were given a choice of a "familiar" or "novel" odour. Time spent investigating each bead was measured.

6.2.4 Blood sampling following restraint stress

Blood sampling for determining the HPA axis response to restraint stress was carried out on rats from cohort 2-4 (Table 6.1). Rats were first surgically cannulated 4 days prior to the restraint stress and blood sampling procedures.

6.2.4.1 Jugular vein cannulation

Surgical cannulation of rats was performed by Dr Paula J Brunton using aseptic technique, as previously described (Brunton and Russell, 2010), with my assistance throughout. The surgical procedure followed closely the protocol by (Thrivikraman et al., 2002), where rats were fitted with a cannula in the right jugular vein. Anaesthesia was first induced in a Perspex chamber with 3% isoflurane in 1.2 L/min oxygen, and then maintained at 2-3% throughout the surgery through inhalation. The rat was then prepared for surgery, with the incision site shaved and cleaned with 70% ethanol. The rat was then placed ventral side up on a heat pad, and the right external jugular vein was exposed following a skin incision rostral to the right clavicle. Two ligatures were then placed loosely around the vein, with the cranial ligature tightened to prevent blood flow from the head into the heart. A V-shaped incision was then made in the vein, into which a sterile silicone cannula (internal diameter, 0.5 mm; outer diameter, 1 mm; filled with sterile heparinised saline, 50U heparin/ml 0.9% saline) was inserted. Once the cannula was inserted 3 cm into the vein, the patency of the cannula was tested, where there should be no resistance to

the withdrawal of blood. A second ligature was then tightened to secure the cannula in position. Once the cannula was secured, it was tunnelled under the skin and exteriorised at the centre of the nape of the neck. The cannula was then closed off with a stainless steel blocker and secured with adhesive taped sutured to the skin. Wounds were closed with suture thread (Ethicon Mersilk) and swabbed with betadine. Rats were administered carprofen (2 mg/kg) intramuscularly as a post-surgery analgesic prior to regaining consciousness, after which time they were monitored closely for signs of pain or distress. Rats were housed individually in specialised open top blood-sampling cages after surgery.

6.2.4.2 Blood sampling and restraint stress

On the day of blood sampling, the jugular vein was connected to a 75cm length of PVC tubing, also filled with sterile heparinised saline, and then left undisturbed for 1 to 2 hr before commencing blood sampling. A blood sample was taken immediately before the rats entered the restraint tubes (0 min time point), and at 15 min, 30 min, 60 min, 90 min, and 120 min from the start of the restraint procedure. Circular restraint tubes (Stoelting, Dublin, Ireland) containing the rat were placed for 30 min in a laminar airflow cabinet, under bright light. The time points at 15 min and 30 min were taken whilst rats were still in the restraint tubes. Blood samples were collected in 1 ml syringes containing 0.02 mL 5% (w/v) EDTA and then transferred to 1.5 mL Eppendorf tubes kept on ice. 0.3 mL of blood was collected per time point, and withdrawn blood was replaced with sterile 0.9% saline after each sample collection. Blood were centrifuged at 1500 g for 20 min at 4^{o}C, and the plasma was removed. Plasma was aliquoted into two tubes and kept at -20^{o}C and -80^{o}C until further use.

6.2.5 Killing and tissue collection

6.2.5.1 Conscious decapitation

In cohort 1, pregnant rats were killed by conscious decapitation at GD20. Dams in the stressed group (7 dams) underwent social stress using the modified resident-intruder-paradigm from GD16 to 20 of gestation in an adjacent room, and were brought to the cull room and killed immediately after the last stressor on GD20 (approximately 11 mins from the induction of stress). Unstressed control dams (7 dams) were removed from the home cage, brought into the cull room and were immediately killed. Killing was carried out in a separate room, and was conducted swiftly by placing the rats in a pliable plastic cone and performing conscious

decapitation using a guillotine. Social stress followed by decapitation was carried out between 10:00 – 14:00 each day.

Trunk blood was collected immediately from pregnant dams, into collection tubes containing 0.5 mL of 0.5% (w/v) EDTA and were kept on ice. Foetuses and placenta were then removed from the uterus and sex was determined based on anogenital distance. Foetuses were decapitated and trunk blood was collected using EDTA-coated capillary collection tubes (Microvette CB 300 µL, Sarstedt, Germany), also kept on ice. Foetal trunk blood were pooled by sex due to the small volumes of blood that could be obtained. The umbilical cord and maternal decidua were removed, and placentae were gently dabbed in clean tissue to remove excess blood. Placentae were either placed into culture media immediately (for placental-conditioned media), or snap frozen in liquid N_2 (for DCF assay), or frozen on dry ice (for ISH). Maternal and foetal brains and liver were collected in Eppendorf tubes and snap frozen in liquid N_2 (for DCF assay and IHC). Both maternal and foetal trunk blood were centrifuged at 1500 g for 20 min at 4^{o}C, and the plasma was removed. Plasma was stored at -20^{o}C until further use. Some of the tissue and plasma samples were shipped to the University of Bristol on dry ice for further analysis.

6.2.5.2 CO_2 overdose

In cohort 2, rats were killed by CO_2 overdose. Rats were placed in a chamber with a rising concentration of CO_2 until respiration ceases. Rats were then decapitated for confirmation of death, and also for brain collection (in the case of Batch 2-2, 2-3; Table 6.1) for *in situ* hybridisation, where they were frozen on dry ice.

6.2.5.3 Cardiac Perfusion-Fixation

Perfusions were carried out by Dr Paula J Brunton and Dr Tom J Phillips from the (University of Bristol, UK). Solutions were prepared the day prior to perfusion, filtered and chilled. PND30 juvenile rats from Batch 2-1 (Table 6.1) were deeply anaesthetised with 3% isoflurane and trans-cardially perfused first with 20 mL heparinised saline, followed by approximately 50 mL 4% paraformaldehyde (PFA; pH 7.3) in PBS, using a butterfly needle connected to a syringe pump. Brains were removed and transferred into 15% sucrose in 4% PFA for 24 hr. Brains were then transferred to 30% sucrose and sent to the University of Bristol in a chilled shipment for immunohistochemistry processing and analysis by Dr Tom J Phillips.

6.2.6 Radioimmunoassays

6.2.6.1 Corticosterone RIA

The corticosterone assay was carried out following the manufacturer's instructions using ^{125}I- corticosterone RIA kits (MP Biomedicals, Eschwege, Germany). The principle of the RIA is based on competitive binding, where a limited amount of anti-corticosterone rabbit antibody and ^{125}I-labelled corticosterone is introduced into the sample. With increasing endogenous corticosterone, less ^{125}I-labelled corticosterone will be able to bind to the available antibody. Goat anti-rabbit gammaglobulin (the secondary antibody) with polyethylene is then added, which precipitates the corticosterone-primary antibody complex. Excess unbound ^{125}I-corticosterone is removed following centrifugation and aspiration, and the amount of radioactivity (cpm) in the precipitate is then measured on a gamma counter. The radioactivity is inversely proportional to the amount of endogenous corticosterone present in the sample.

Corticosterone RIA were carried out on the maternal/foetal plasma (cohort 1) and male and female samples (cohort 2-3; Table 6.1). All samples from cohort 1 were analysed in one RIA while males and females from Batch 2-3 were analysed on separate days. Briefly, plasma was diluted 1:200 with steroid diluent buffer.100 µL of samples, standard calibrants, and low and high concentration quality controls were added to assay tubes in duplicate. 100 µL of ^{125}I-corticosterone and 100 µL of anti-corticosterone antibody was then added to each tube. Following a 2 hr incubation, a precipitant solution was added. After centrifugation and aspiration of supernatants, the radioactivity of the pellets (in cpm) were counted in a gamma counter.

Additionally, three other standards were also prepared in duplicate and their radioactivity measured, (i) total count standard: where an aliquot of ^{125}I-corticosterone was used to measure total cpm, (ii) non-specific binding: where only the ^{125}I-corticosterone and secondary antibody were added, (iii) B_{max}: which consists of only ^{125}I-corticosterone, primary and secondary antibody but no sample, allowing us to determine maximum binding. An inverse linear regression curve, plotted using percentage binding (cpm obtained for sample/ cpm obtained for B_{max}) against the known concentrations, was then generated on AssayZap software (Biosoft, Cambridge, UK). The amount of corticosterone present in the sample was then intrapolated. The range of detection was 7.7 ng/ml to 1000 ng/ml, and cross-

reactivity was reported by the manufacturer to be <0.34% with any other steroids commonly found in plasma.

6.2.6.2 ACTH RIA

Total ACTH in the plasma of male and female rats in Batch 2-4 (Table 6.1) were measured using the MP Biomedicals ACTH Double Antibody RIA Kit (Cat. 07106102) as per the manufacturer's instructions, by Dr Paula J Brunton. The principle of the ACTH RIA is similar to that of the corticosterone RIA, with ^{125}I-ACTH as the antigen and anti-ACTH as the corresponding primary antibody. 100 µL of undiluted plasma sample kept in -80^{o}C was used, and samples were run in duplicate.

6.2.7 Oxidative stress assay

Levels of ROS were measured in maternal, placental and foetal tissues (cohort 1) using the 2′,7′-dichlorofluorescein diacetate (DCF-DA) assay. In this assay, a two-step reaction occurs to generate the fluorescent 2′,7′-dichlorofluorescein DCF, which is a surrogate marker for ROS levels in the cell (Kalyanaraman et al., 2012). Upon permeating cells, DCF-DA is first hydrolysed by intracellular esterases into form the intermediate DCFH. In the second step of the reaction, DCFH is subsequently oxidised by ROS to form the fluorescent DCF.

Tissues collected from cohort 1 were shipped on dry ice to the University of Bristol, where the DCF assay was performed by Dr Tom J Phillips, as previously described (Phillips et al., 2017). Briefly, the foetal brain, liver, placenta, maternal brain and liver were cut sagittally (10 µm) on the cryostat and thaw mounted on slides. Sections were then incubated with 20 µM DCF-DA solution (Sigma-Aldrich) in Hanks' Balanced Salt solution (HBSS) at 37^{o}C in a humidifying chamber for 15 min, before counter-staining with DAPI for cell nuclei. Slides were then immediately imaged using a confocal microscope where fluorescent DCF levels were quantified (excitation: 495 nm, emission: 529 nm).

6.2.8 *In situ* hybridisation

In situ hybridisation was carried out to probe for CRH mRNA in the central amygdala (CeA) of PND30 (Batch 2-2; Table 6.1) and 12 week old male offspring (Batch 2-3; Table 6.1), and 11β-HSD2 mRNA in the placenta (Batch 1).

Tissue sectioning: Frozen brains and placenta were mounted (but not embedded) on OCT (Tissue-Tek) and 16µm sections were cut using a cryostat at -19^{o}C (Leica CM1850), then thaw-mounted on Polysine adhesion slides (ThermoScientific). Brains from 12 week male offspring (cohort 2-3) were cut by MSc student Joanne Palsma. In each case, every 4th section was collected on each slide, and each slide consisted of 4 (12 week males) or 5 (PND30 males) sections. The amygdaloid complex was identified in reference to a rat brain atlas (Paxinos and Watson, 2007). Marker sections were collected on gelatine subbed slides (1% gelatin with chromium), and fixed with acetic alcohol fixative (4% w/v formaldehyde and 5% v/v acetic acid in ethanol) and stained with 1% toluidine blue for visualisation. Tissue sections on slides were stored in -80^{o}C until *in situ* hybridisation.

In situ hybridisation: In situ hybridisation was carried out as described in Chapter 2, with specific conditions for each probe detailed in Table 6.2. Briefly, plasmids containing cDNA of the gene of interest were first linearised with the respective restriction enzymes. The linearised cDNA was then used to synthesise the sense and antisense ^{35}S–UTP labelled riboprobes by RNA transcription, catalysed with the specific RNA polymerases. Probes were then hybridised with tissue sections on slides overnight at 55^{o}C, which had been fixed and pre-hybridised beforehand (see Chapter 2). Post hybridisation washes, which consisted of 2X SSC (RT for 30 min, once) followed by 0.1X SCC (60^{o}C for 50 min, three times), were then carried out. Hybridised probes were then exposed to radiosensitive autoradiographic emulsion in 4^{o}C (Table 6.2) before development and counterstaining. Compositions of buffers and optimal conditions for reactions are described in Chapter 2.

Target mRNA	Original plasmid source	Restriction enzymes	Plasmid vector; Size of insert	RNA Polymerase	Exposure time
CRH	(Harris et al., 2001)	AS: XbaI S: HindIII	518 bp	AS: T3 S: T7	25 days
11β - HSD2	(Leckie et al., 1995)	AS: SphI S: SalI	750 bp	AS: SP6 S: T7	4 weeks

Table 6.2: Conditions for *in situ* hybridisation. AS: Antisense, S: Sense. Hybridisation was carried out at 55°C for both probes, while post-hybridisation washes consisted of the first step of 2x SSC at RT for 30min, followed by 0.1x SSC at 60°C for 50 min three times. Both probes were kindly donated by Prof Megan Holmes (University of Edinburgh).

Data analysis and quantification: Quantification of hybridisation was carried out by positive cell counts at 20X magnification. Cells were considered positive when the silver grains were imposed on the cell at a density 5x higher than that of the background. Counts were averaged across 4 or more sections for the CeA bilaterally, and across 5 sections for the placental junctional zone.

6.2.9 Immunohistochemistry of PND30 brains

Perfused PND30 brains (Batch 2-1, Table 6.1) were processed for immunohistochemistry by Dr Tom J Phillips. Three brains per group per sex were used.

Tissue sectioning, fixing and permeabalisation: Cryostat sections (12 µm) were cut coronally and mounted on slides as contiguous triplicates. For $GABA_{A\alpha1}$, $GABA_{A\alpha2}$, $GABA_{B1}$, parvalbumin, and MAP2 staining for dendrites, sections were fixed in cold methanol (-20°C) for 10 min. For GluN1 staining, sections were first fixed in 2% PFA, and then permeabilised in 0.3% Triton X-100 in PBS for 15 min.

Immunostaining: Sections were blocked with 5% goat serum, 0.3% Triton X-100 in PBS for 2 hr at 4°C to reduce non-specific binding. Sections were then incubated overnight at 4°C with primary antibody in PBS with 1% BSA and 0.3% Triton X-100 (except for GluN1 staining, which was incubated for two overnights). Primary antibodies were used against MAP2 (1:500, Abcam #ab32454; rabbit polyclonal), parvalbumin (1:500, Abcam #ab11427; rabbit polyclonal), $GABA_{A\alpha1}$ (1:500, Abcam

#ab33299; rabbit polyclonal), $GABA_{A\alpha2}$ (1:200, Abcam #ab193311; mouse monoclonal), $GABA_{B1}$ (1:500, Abcam #ab55051; mouse monoclonal) or GluN1 (1:500, Merck Millipore #ab9864; rabbit monoclonal). After sections were washed, sections were incubated with secondary antibody for 2 hr at 4°C. Secondary antibodies were either anti-rabbit IgG (Alexa Fluor 555), anti-mouse IgG (Alexa Fluor 488 or 568) from Thermo Fisher Scientific, all prepared at 1:500. Vectashield Mounting Medium (with DAPI counterstain to visualise nuclei)(Vector Laboratories, USA) was used to mount coverslips.

Data analysis and quantification: 5 sampling views were taken in each hemisphere for each brain region in each of the 3 sections, resulting in 30 sampling views per rat, except for GluN1, 10-12 images were taken for each region. Sections were visualised on a SP5II confocal microscope (SP5II, Leica) at 40X magnification. Images were quantified on ImageJ, where they were converted to greyscale and the total pixel number was determined, subsequently subtracted from the background to obtain a mean grey value.

6.2.10 E18 cortical cultures experiments

6.2.10.1 Preparation of placenta conditioned media

Conditioned tissue culture media were prepared using fresh rat placentae isolated from cohort 1. Immediately after collection, whole placentae were incubated individually at 37°C in 21% O_2 and 5% CO_2, in 1.5 mL of warmed neurobasal culture medium in 12 well plates. Neurobasal culture medium (Gibco) was supplemented with 1X B-27 supplement (Gibco), 2 mM L-glutamine and 250 µM penicillin-streptomycin (ThermoFisher), prepared in sterile conditions. After 24 hr, the media around the explants was collected using a syringe, filtered through a disk filter (0.22 µm, MF-Millipore) into Eppendorf tubes, and frozen on dry ice. Placental-conditioned media was shipped to the University of Bristol on dry ice and was kept at -80°C until further use.

6.2.10.2 Cortical cultures

Preparation of cortical cultures were carried out by Dr Tom J Phillips at the University of Bristol. Cortical cultures were prepared from dissociated rat embryonic day 18 cortical tissue and grown on glass coverslips as described previously (Curtis et al., 2014), in neurobasal culture medium (section 6.2.10.1). Placenta conditioned

medium (section 6.2.10.1) or foetal plasma (section 6.2.5.1) was then applied to the cortical cultures for 6 days starting from the 12th day of *in vitro* culture.

6.2.10.3 Immunocytochemistry of cortical cultures

Cortical cultures were investigated for GluN1, $GABA_{A\alpha1}$, $GABA_{A\alpha2}$, $GABA_{B1}$ receptors, parvalbumin, and MAP2 (a dendritic marker). Cortical cultures were fixed in supercold methanol (−20^{o}C) and blocked with 5% BSA, 5% normal goat serum in PBS for 30 min. They were incubated with primary antibodies (same as in section 6.2.9) overnight at 4^{o}C, the secondary antibody (same as in section 6.2.9) for 2 hr at room temperature under minimal light conditions, washed with PBS and similarly mounted in DAPI mounting media. Five images per coverslip were taken on a confocal microscope (SP5II, Leica). Images were taken at 64x magnification (with oil) and quantified similarly using ImageJ.

6.2.11 LC-MS quantification of steroids in maternal plasma

LC-MS quantification of steroids in maternal plasma was carried out as described in Chapter 2 and 3. 100 µL of neat maternal plasma from cohort 1 (stored in -20^{o}C) was processed as described in Chapter 3, along with 7 calibration standards. Plasma samples were extracted twice with methanol/1% formic acid, and underwent C18 solid phase extraction. All samples were derivatised at the same time using 1 mg/ml Girard's T reagent, in methanol/ 0.2% formic acid, dried and reconstituted in 50% methanol. LC-MS detection of steroids (DOC, DHDOC, THDOC, progesterone, DHP, allopregnanolone, pregnenolone, testosterone) were carried out with the transitions outlined in Table 3.4.

6.2.12 Data analysis and statistics

Two-way ANOVAs were carried out using R-Studio or PRISM 6.0 (for DCF assay and IHC studies only), with stress and maternal antioxidant treatment as the main factors investigated. Males and females were analysed separately. Pairwise comparisons were carried out using Student's-Newman-Keuls test or Bonferroni-corrected pairwise comparisons (for DCF assay and IHC studies only) to determine significant differences between groups. In all cases, asterisks represent differences between control and PNS groups (where * $p<0.05$, ** $p<0.01$, *** $p<0.001$), while hashes represent differences between vehicle and MitoQ-NP treated groups (where # $p<0.05$, ## $p<0.01$, ### $p<0.001$).

6.3 RESULTS

6.3.1 Corticosterone concentrations following stress were not affected by MitoQ-NP administration

Maternal: As expected, a main effect of stress was observed on the maternal plasma corticosterone concentrations ($F_{1,23}$ = 25.2, p<0.001; Fig 6.6A). There were no main effects of drug treatment, nor any interactions. Both vehicle and MitoQ-NP treated stressed groups had significantly greater maternal corticosterone concentrations compared to their respective control groups. MitoQ-NP administration to the pregnant dam did not result in different plasma corticosterone concentrations compared to controls (Control/Vehicle vs Control/MitoQ-NP, p=0.37).

Foetal: There were no differences in plasma corticosterone in all four groups for both the male (Fig 6.6B) and female foetuses (Fig 6.6C). The potential actions of maternal MitoQ-NP administration were thus unlikely to occur through the alteration of circulatory corticosterone concentrations.

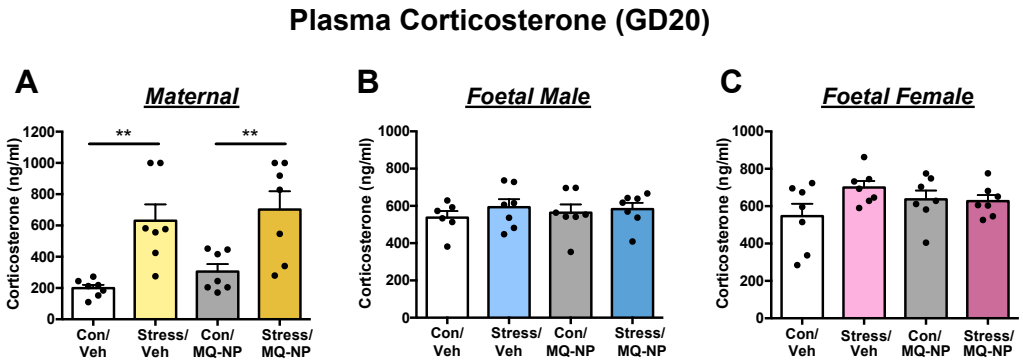


Figure 6.6: Plasma corticosterone concentrations in the pregnant dam and foetuses, measured by radioimmunoassay. At GD20**,** corticosterone concentrations were significantly greater in stressed dams regardless of antioxidant treatment (A). There were no differences in all four groups for pooled plasma corticosterone in male foetuses (B) or for females foetuses (C). n=7 per group except for foetal male stress/vehicle group where n=6. Asterisks represent significant differences between controls and stressed groups in SNK multiple pairwise comparisons, where ** p<0.01. Con: Control, MQ-NP: Veh: Maternal vehicle treatment, MQ-NP: Maternal MitoQ-NP treatment.

6.3.2 Social stress was associated with greater ROS production in the maternal compartment and placenta, which could be blocked by administration of MitoQ-NP

There were significant main effects of stress, treatment and stress x treatment interactions on DCF levels (reflecting ROS levels) in the placenta, maternal brain and maternal liver (Fig 6.7A-C). There were significant differences between the vehicle-treated control and stressed groups in all three tissues, where stressed groups had significantly greater DCF levels, indicating the presence of greater ROS production. Therefore, social stress resulted in greater ROS production in the maternal compartment and placenta.

MitoQ-NP treated stressed groups had significantly lower DCF levels as compared to vehicle-treated stress groups. DCF levels in the placenta, maternal brain and liver of MitoQ-NP treated stressed groups were also not different from that of vehicle or MitoQ-NP treated control groups, suggesting they had similar ROS content as the control groups (Fig 6.7A-C). Therefore, administration of maternal MitoQ-NP could abrogate the increased ROS production in placenta, maternal brain and maternal liver following stress, indicating the efficacy of the antioxidant treatment in the maternal compartment.

Despite MitoQ-NP not crossing over into the foetal compartment, there was a significant main effect of stress and a stress x treatment interaction on DCF levels in the foetal liver (Fig 6.7D). DCF levels in the vehicle-treated stressed group were higher than that of the vehicle-treated control group. The MitoQ-NP treated stressed group had lower DCF levels than the non-treated stress group, but DCF levels were still greater than the vehicle-treated control group, indicating greater ROS content. The administration of MitoQ-NP to the dam attenuated ROS production in the stressed foetal liver.

Neither maternal stress nor maternal MitoQ-NP administration were associated with any ROS changes in the foetal brain (Fig 6.7E).

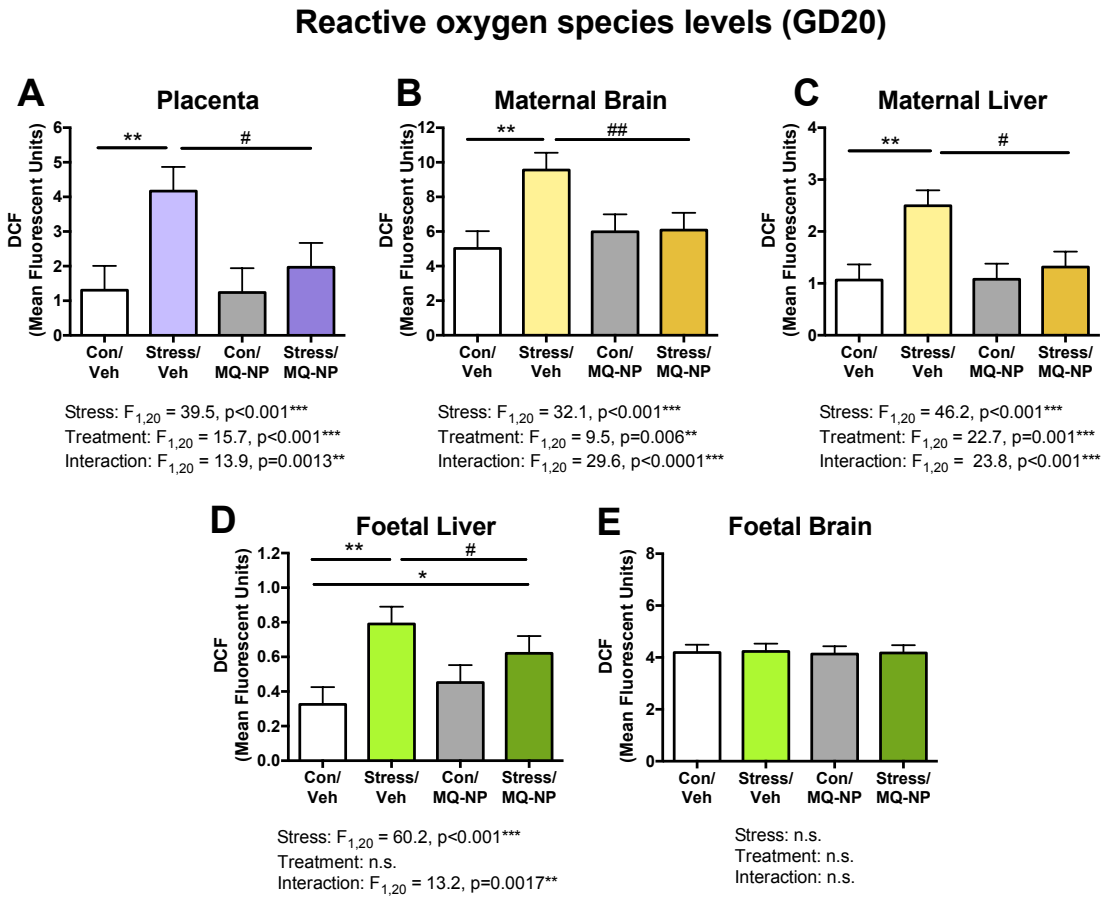


Figure 6.7: Reactive oxygen species content in placenta, maternal and foetal tissues following prenatal stress and maternal MitoQ-NP treatment. At GD20, elevated ROS was observed in the stressed rats in the placenta (A), maternal brain (B), maternal liver (C), foetal liver (D) but not the foetal brain (E). Maternal MitoQ-NP treatment abrogated the elevated ROS levels in all these tissues, although the rescue in the foetal liver (D) seemed to be partial. Oxidative stress was measured by quantifying mean fluorescent intensity of DCF and the assay was carried out by Dr Tom Phillips of University of Bristol. Asterisks represent differences between control and PNS groups (where ** p<0.01), while hashes represent differences between vehicle and MitoQ-NP treated groups (where # p<0.05). Con: Control, Veh: Maternal vehicle treatment, MQ-NP: Maternal MitoQ-NP treatment, DCF: Dichlorofluorescin

6.3.3 Maternal MitoQ-NP administration rescued the anxiety phenotype in the adult male offspring

Anxiety testing (LDB and EPM) was carried out in the offspring of pregnant rats from cohort 2 at about 9 weeks of age. Anxious behaviour was characterised by decreased exploration time in the light box in the LDB and a lower frequency of entry into the open arms of the EPM, and an increased latency to enter the light box in the LDB.

6.3.3.1 Light-dark box (LDB)

Males: There was a significant main effect of PNS status ($F_{1,27} = 4.50$, p=0.04) and a PNS status x treatment interaction that was close to significance ($F_{1,27} = 2.77$, p=0.11) on the amount of time males spent in the light box (Fig 6.8A). Post-hoc analyses showed that vehicle-treated PNS males spent significantly less time exploring the light box than control males (p=0.011), as expected (Brunton and Russell, 2010). PNS males with maternal MitoQ-NP treatment however, spent significantly more time in the light box compared to the PNS males from vehicle-treated dams (p=0.027), and time spent in the light box was not different from that of the control males from vehicle-treated dams. This indicates that maternal treatment with MitoQ-NP normalised anxiety-like behaviour induced by maternal stress in the PNS males to control levels. A similar pattern of behaviour was observed for the number of entries into the light box (Fig 6.8B), however, this was not significant.

PNS males from vehicle-treated dams tended to have a greater latency to enter the light box as compared to the controls (Fig 6.8C), but this was not observed for the PNS males from matermal MitoQ-NP treated dams. This observation however, did not reach statistical significance using a two-way ANOVA.

There was an additional main effect of maternal MitoQ-NP treatment ($F_{1,27} = 5.20$, p=0.03) on total distance travelled within the arena (Fig 6.8D). PNS males with maternal MitoQ-NP treatment had significantly greater exploratory activity in both light/dark boxes, as compared to PNS males from vehicle-treated dams (p=0.039). This measure was not different between the vehicle-treated control and PNS males.

Females: There were no significant differences across any of the four groups in all parameters investigated in the light-dark box, as expected (Fig 6.8F-I) (Brunton and Russell, 2010).

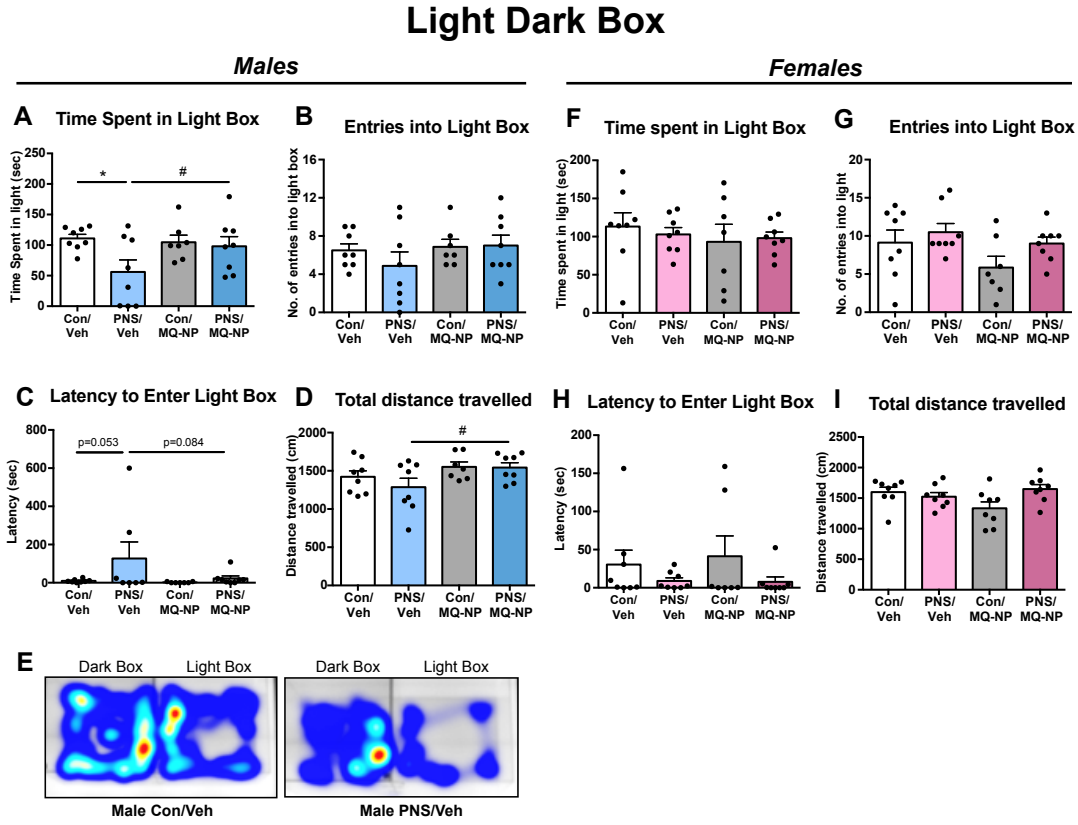


Figure 6.8: Effects of prenatal stress and maternal MitoQ-NP administration on anxiety-like behaviour in the light dark box. Results from the male offspring are in the left panel (A–E) and females in the right panel (F- I). (A) Male PNS offspring spent less time in the light box than control offspring, and this was rescued by the maternal administration of MitoQ-NP. (B) No significant differences were observed in the number of entries into the light box. (C) Some PNS offspring showed an increased latency to enter the light box, but group means were not significantly different when analysed by two-way ANOVA. (D) Total distance travelled was significantly greater in PNS male offspring with maternal MitoQ-NP administration as compared to the vehicle-treated PNS male offspring. (E) Representative heat maps depicting differential amount of time male control and PNS offspring spent exploring the dark and light areas of the LDB. No difference was observed in any of the four groups for the females, in the time spent in light box (F), entries into light box (G), latency to enter light box (H) and total distance travelled. n=8 per group except for male PNS/MitoQ-NP group, where one outlier was removed. Asterisks represent differences between control and PNS groups (where * $p<0.01$), while hashes represent differences between vehicle and MitoQ-NP treated groups (where # $p<0.05$). Con: Control, PNS: Prenatal stress, Veh: Maternal vehicle treatment, MQ-NP: Maternal MitoQ-NP treatment.

6.3.3.2 Elevated plus maze (EPM)

Males: A similar finding as for the LDB was observed in the EPM, where there was a main effect of PNS status ($F_{1,26}$ = 7.57, p=0.01) and a PNS status x treatment interaction that was close to significance ($F_{1,26}$ = 3.36, p=0.07) on the frequency of entries into the open arm (Fig 6.9A). PNS males had a significantly lower frequency of entering the open arms as compared to control males (p=0.0042; Fig 6.9E), and this seemed to be normalised in the maternal MitoQ-NP treated PNS groups (p=0.06). In terms of number of entries into open arms, there was a main effect of PNS status ($F_{1,26}$ = 4.82, p=0.03) but no interaction was observed (Fig 6.9C). Vehicle-treated PNS males made significantly fewer entries into the open arms as compared to vehicle-treated controls, while maternal MitoQ-NP treatment seemed to reverse this effect (p=0.09). A similar trend was observed for time spent in open arms (Fig 6.9B), although this did not reach significance. There were no differences across all 4 groups for total entries into all arms (Fig 6.9D).

Females: There were no significant differences across all four groups for the time spent (Fig 6.9G) or the frequency to enter the open arms (Fig 6.9F). There was a significant PNS status x treatment interaction ($F_{1,28}$ = 9.25, p=0.005) for total entries into all arms. PNS females seemed to have a decreased exploration in the EPM (Fig 6.9I), where they had significantly lower entries into all arms as compared to the control groups (p=0.0269). Maternal MitoQ-NP treated PNS females, however, had significantly greater total entries as compared the vehicle-treated PNS females (p=0.0092; Fig 6.9I), which indicated that maternal MitoQ-NP adminstration could have increased the total exploration in the EPM in the female PNS offspring.

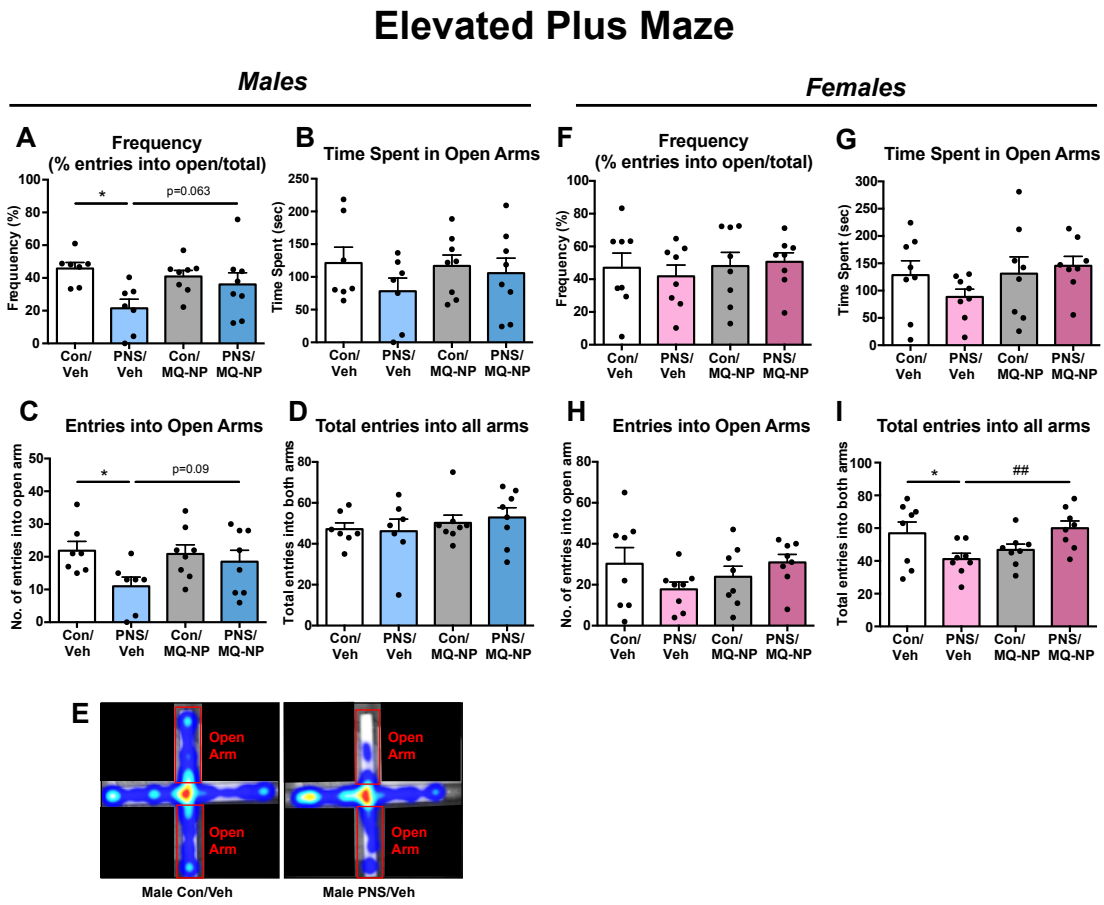


Figure 6.9: Effects of prenatal stress and maternal MitoQ-NP administration on anxiety behaviour on the elevated plus maze in male and female adult offspring. Results from male offspring are presented in the left panel (A – E) and females presented in the right panel (F- I) (A) Male PNS offspring had a lower frequency of entering open arms as compared to control offspring, and this seemed to be rescued by the maternal administration of MitoQ-NP. (B) Male PNS offspring made fewer entries into the open arms as compared to control offspring, and this seemed to be rescued by the maternal administration of MitoQ-NP. (C) No differences in the time spent in the open arms across all groups for male offspring. (D) Total entries into all arms was not different across all groups for the male offspring. (E) Representative heat maps depicting differential amount of time male control and PNS offspring spent exploring the open and closed arms of the EPM. In females, there were no differences observed in all four groups for females, frequency of entries into open arms (F), number of entries into open arms (G) and time spent in open arms (H). (I) PNS females, however, had decreased total entries into both arms, and this was normalised by maternal MitoQ-NP administration. Asterisks represent differences between control and PNS groups (where * $p<0.05$), while hashes represent differences between vehicle and MitoQ-NP treated groups (where ## $p<0.01$). Con: Control, PNS: Prenatal stress, Veh: Maternal vehicle treatment, MQ-NP: Maternal MitoQ-NP treatment.

6.3.4 Effects of PNS and maternal MitoQ-NP treatment on sucrose preference in the adult offspring

A two-way ANOVA did not reveal any significant differences in the percentage of sucrose consumption across all groups in the combined data from across two days of testing (Fig 6.10A and 6.10D). In males (Fig 6.10A), there was no difference in the intake of sucrose across all groups. Most male rats, regardless of PNS status or maternal MitoQ-NP treatment, showed a strong preference for sucrose as compared to water, except for one rat per group which seemed to have either no preference (one PNS/veh rat) or prefer water (one rat for each of the MitoQ groups). In females (Fig 6.10D), a two-way ANOVA did not reveal any significant preferences, but the PNS/Veh group seemed to exhibit a binging effect where all rats in that group exhibited a strong preference for sucrose over water.

The data was also broken down by testing days. In male offspring, there were no differences in sucrose consumption patterns across all four groups on both testing day 1 (Fig 6.10B) and day 2 (Fig 6.10C). In the female offspring, there was a PNS status x maternal MitoQ-NP treatment interaction on testing day 1 ($F_{1,28}$=4.81, p=0.037; Fig 6.10E). The average sucrose consumption in the control group was close to being significantly lower than that of the PNS group (p=0.059) and control/MitoQ-NP group (p=0.079) on testing day 1. This trend was not observed when compared to the PNS/MitoQ-NP group, and it was also not present on testing day 2. On testing day 2, although the two-way ANOVA was not significant ($F_{1,28}$=2.63, p=0.12; Fig 6.10F), pairwise comparisons showed a significant difference between the percentage of sucrose consumed between the vehicle-treated PNS group and the maternal MitoQ-NP-treated PNS group (p=0.048).

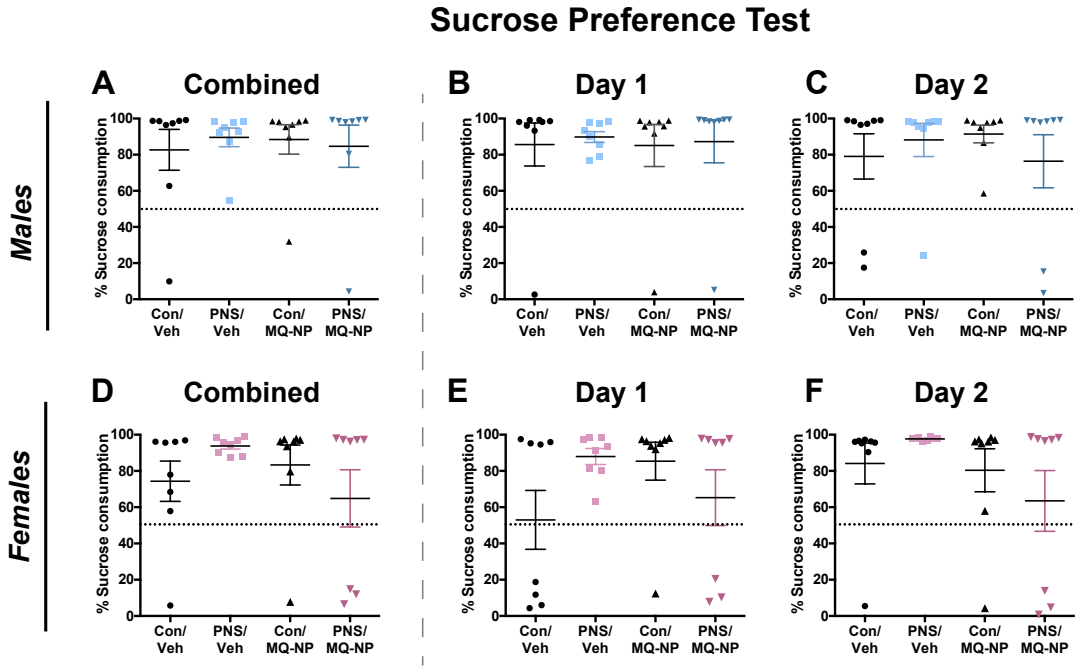


Figure 6.10: Sucrose preference test in male and female offspring. Two-way ANOVA did not reveal any significant differences in the groups in terms of percentage sucrose consumption. Data points above the dotted line (50%) indicated that rats preferred sucrose, while data points below the dotted line indicated that rats preferred water. The bottles were switched between Day 1 and Day 2 to ensure that there were no side preferences "Combined" (A and D) refers to consumption over the 48 hr period, while "Day 1" and "Day 2" refers to consumption on the respective testing days (24 hr each). Con: Control, PNS: Prenatal stress, Veh: Maternal vehicle treatment, MQ-NP: Maternal MitoQ-NP treatment.

6.3.5 Maternal MitoQ-NP administration, but not PNS, altered adult offspring behaviour during the forced swim test

Males: In the male offspring, there was a main effect of maternal antioxidant treatment on floating ($F_{1,27}$ = 9.57, p=0.0045; Fig 6.10A) and swimming ($F_{1,27}$ = 7.88, p=0.009; Fig 6.10B) behaviours, but not climbing behaviours. No main effect of PNS status was observed for any of the behaviours measured. Post-hoc SNK testing showed that floating behaviour occurred less frequently for offspring from MitoQ-NP treated dams as compared to the offspring from vehicle-treated dams, regardless of PNS status (Fig 6.10A).

Females: In the female offspring, there was a main effect of maternal antioxidant treatment on climbing behaviours only ($F_{1,27}$=5.88, p=0.022; Fig 6.10F). SNK multiple pairwise comparisons showed that female control offspring from MitoQ-NP treated dams had increased frequency of climbing bouts as compared to control offspring from vehicle-treated dams, whilst a trend for an increase was observed in the PNS groups. Similar to males, no effect of PNS status was observed for any of the behaviours in the female offspring.

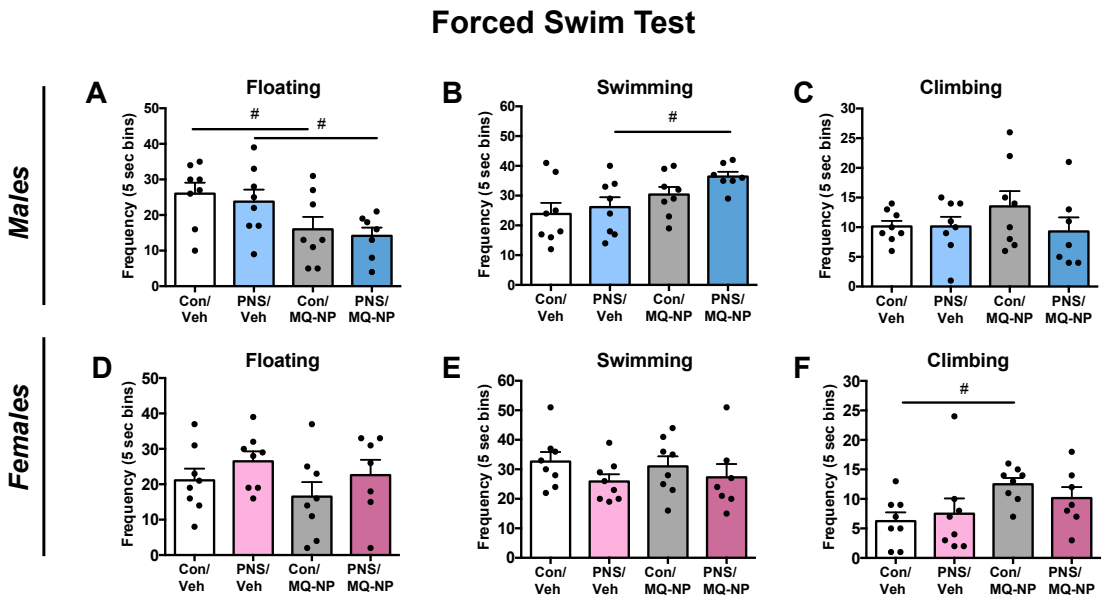


Figure 6.11: Maternal MitoQ-NP administration differentially altered male and female offspring behaviours in the forced swim test. Frequency of floating, swimming and climbing behaviours did not change with PNS for both the male (A-C) and the female (D-F) offspring. Maternal MitoQ-NP treatment resulted in less floating behaviours (A) and increased swimming behaviours (B) in males, but did not alter climbing behaviour (C). Maternal MitoQ-NP treatment increased climbing behaviour in females (F) but did not alter floating (D) nor swimming (E) behaviours. Hashes represent an effect of drug administration in the post-hoc SNK test, where #$p<0.05$. N=8 per group. Con: Control, PNS: Prenatal stress, Veh: Maternal vehicle treatment, MQ-NP: Maternal MitoQ-NP treatment.

6.3.6 Maternal MitoQ-NP administration does not affect performance in the social olfactory memory test in the female offspring

There was a main effect of PNS status that was close to significance on the preference for novel odour ($F_{1,29} = 3.81$, p=0.06, Fig 6.12B). PNS females seemed to show a deficit in social olfactory memory, although this did not reach significance.

When the actual time spent interacting with the beads was investigated within each group (Fig 6.12C), in the control/veh group, time spent investigating familiar and novel beads was significantly different (p=0.043), and more time was spent investigating the novel object. In the control group with maternal antioxidant treatment, a similar trend was observed (p=0.179). In the PNS groups however, there were no differences in time spent investigating either objects, further indicating a deficit in social memory in PNS offspring that was not rescued by maternal MitoQ-NP administration.

There were no statistically significant differences in the investigation time of the "familiar" bead during the exposure phase, 3 hrs before the test phase (Fig 6.12A), indicating that any possible differences between groups during the test phase were probably not due to the lack of interaction with the object during the exposure phase.

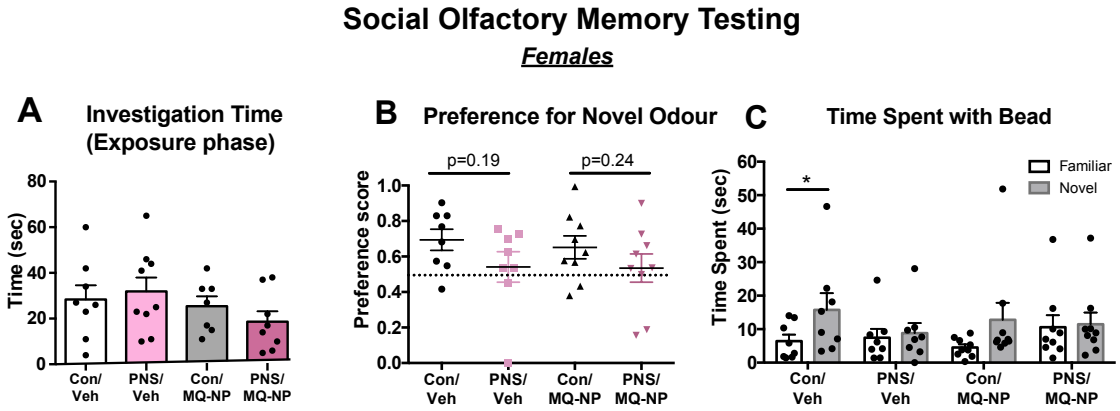


Figure 6.12: Social/olfactory memory testing in female offspring. (A) Investigation time of the "familiar" bead during the 4 min exposure phase. (B) Results during the 'choice' phase (3 hr later): Data presented as a preference score for the novel odour. The horizontal line at 0.5 indicates chance level, where there was no preference for either the novel or the familiar odour. Control groups seemed to show a greater preference for the novel odour, as compared to the respective PNS groups (regardless of maternal antioxidant treatment), although this did not reach significance. (C) Results during the 'choice' phase (3 hr later): Data presented as time spent with familiar/novel beads, where the Control/Veh group showed significantly greater time spent investigating the novel beads as compared to the familiar beads. This was not observed in the other groups, although a trend was observed for the Con/MitoQ NP group. *p<0.05, paired Student's t-test.

6.3.7 Maternal MitoQ-NP treatment had no effect on HPA axis responses to stress in the adult offspring

There were no significant differences detected in plasma ACTH and corticosterone concentrations in all four groups, in both sexes, before, during, and following 30 min of restraint stress (Fig 6.13).

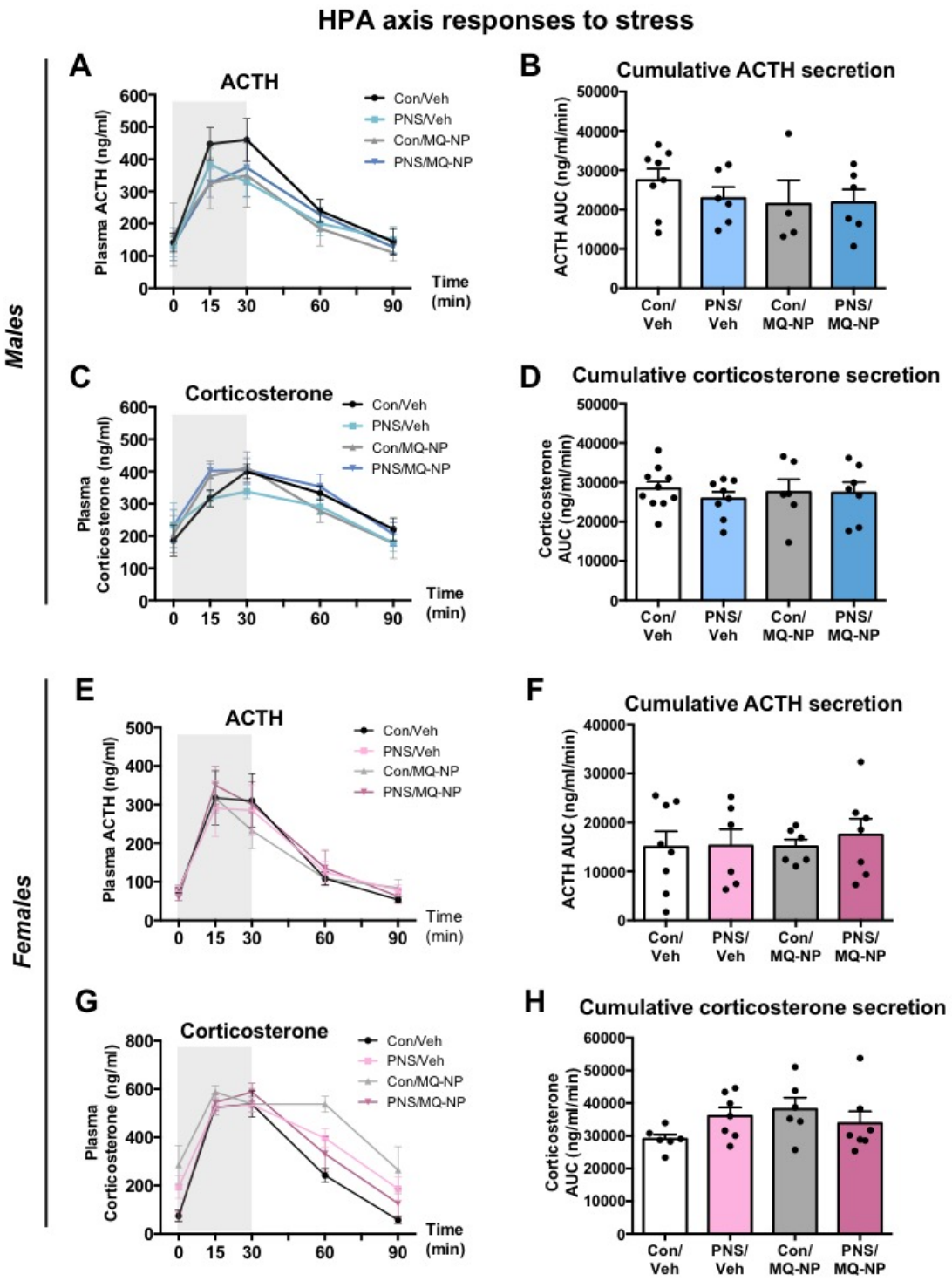


Figure 6.13: ACTH and corticosterone responses to 30 min restraint stress. A basal blood sample was taken (0 min) before rats were restrained, and blood samples were taken at 15, 30 (before being removed from restraint tube), 60, and 90 min after the start of restraint, via a jugular vein cannula. The period of restraint is represented by the shaded grey bar (A, C, E, G). No differences in corticosterone secretion was present in any of the groups. Con: Control, PNS: Prenatal stress, Veh: Maternal vehicle treatment, MQ-NP: Maternal MitoQ-NP treatment.

6.3.8 Maternal MitoQ-NP adminstration normalised increased CRH mRNA expression in the central amygdala in male offspring

An increase in CRH mRNA expression is associated with the anxiety phenotype in males (Brunton and Russell, 2010), and here this was also observed for both juvenile (PND 30; Fig 6.14B) and adult PNS offpsring (Fig 6.14A).

In the adult offspring, there was a main effect of PNS status ($F_{1,25}$ = 12.07, p=0.002), a main effect of maternal MitoQ-NP adminstration ($F_{1,25}$ = 4.99, p=0.035) and also a significant PNS status x maternal treatment interaction ($F_{1,25}$ = 6.79, p=0.015; Fig 6.14A). Post-hoc SNK testing in adult male offspring showed that the PNS/Veh group had significantly higher CRH mRNA expression as compared to the Con/Veh group (p<0.001). PNS males with maternal MitoQ-NP adminstration had significantly lower CRH mRNA expression levels as compared to PNS males without maternal MitoQ-NP adminstration (PNS/MitoQ-NP vs PNS/Veh, p=0.003), and CRH mRNA levels were also not different from those in control rats, indicating that maternal MitoQ-NP adminstration normalised the PNS-induced increase in CRH gene expression in the central amygdala (Fig 6.14A).

In the juvenile offspring, a similar pattern was observed, however, the two-way ANOVA did not reach significance (PNS status x maternal MitoQ-NP adminstration interaction, $F_{1,16}$=3.41, p=0.08; Fig 6.14B).

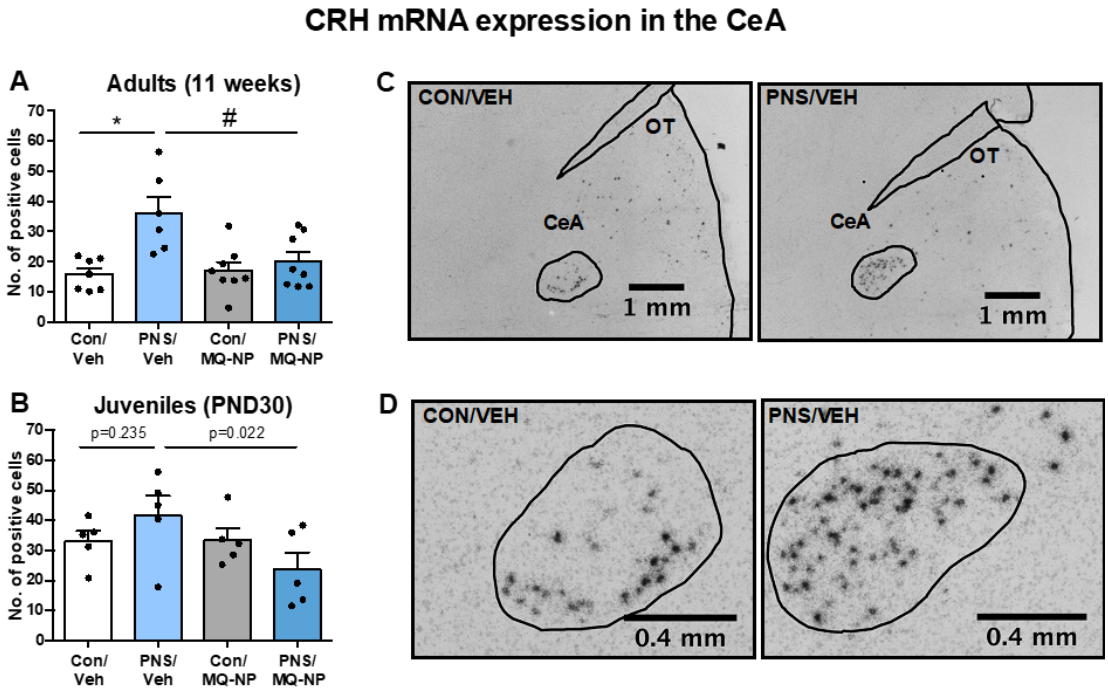


Figure 6.14: CRH mRNA expression in the central amygdala (CeA) of male offspring. (A) CRH mRNA expression in the CeA was greater in 11 week old PNS/veh offspring as compared to the respective control group, and this normalised with maternal MQ-NP treatment. (B) CRH mRNA expression also tended to be greater in PND30 juvenile PNS/veh offspring as compared to the respective control group, although this was not significant on the two-way ANOVA and post-hoc SNK pairwise comparisons. Group numbers: n=6-8 for adult offspring, n=5 for juvenile offspring (C) Representative image of the CeA and its anatomical position with respect to the optic tract (OT) at 2x magnification, in PND30 juvenile male rat brain sections. (D) Representative image of Male Con/Veh and Male PNS/Veh CeA at 10x magnification, showing positive cells with silver grain staining, in PND30 juvenile male rat brain sections. Con: Control, PNS: Prenatal stress, Veh: Maternal vehicle treatment, MQ-NP: Maternal MitoQ-NP treatment.

6.3.9 PNS and maternal MitoQ-NP treatment altered neurochemical markers in the juvenile offspring brain in a region- and sex-dependent manner

Several other neurobiochemical markers were also quantified in the offspring brain, and these were carried out by Dr Tom J Phillips at the University of Bristol. Graphs with statistical analyses are attached in Appendix D, and a summary of the changes are presented in Figure 6.15. In general, there was a region- and sex-dependent decrease in the expression of several receptors and neuronal markers (e.g. parvalbumin for GABA producing cells, MAP2 for dendrites), and these deficits were partially or completely normalised by maternal MitoQ-NP treatment.

Across the board, $GABA_{A\alpha1}$, $GABA_{A\alpha2}$ and $GABA_{B1}$ expression and GluN1 expression were observed to be lower in the PNS offspring, although the changes were also dependent on brain region and sex. For instance, in the hippocampus, the CA3 region seemed to be largely spared from the GABA receptor expression abnormalities, whilst the hippocampal CA1 and CA2 regions seemed to be more susceptible to decreases in $GABA_{A\alpha1}$, $GABA_{A\alpha2}$ and $GABA_{B1}$ receptor subunit expression in the PNS offspring. Although there were modest sex differences in receptor subunit expression in the hippocampus of PNS offspring, this difference was starker in the BLA, where male PNS offspring displayed significanty lower $GABA_{A\alpha1}$ and $GABA_{A\alpha2}$ subunit expression as compared to controls, but females were unaffected. Similarly, dendritic length of neurones and PV+ cell counts seemed to be decreased predominantly in the PNS males as compared to the control males, however this observation was not observed in females, except for in the hippocampal CA1 region.

Maternal MitoQ-NP administration could rescue some deficits associated with PNS, especially in the BLA. In the hippocampus, this normalisation seemed to be sub-region specific, where maternal MitoQ-NP treatment had an ostensibly larger effect in the CA1 region than in the CA2. As for dendritic length and PV+ cell counts, a partial rescue was also observed, with normalisation in certain regions following MitoQ-NP adminstration (e.g. hippocampal regions CA2 and CA3) but less so in other regions (e.g. hippocampal CA1).

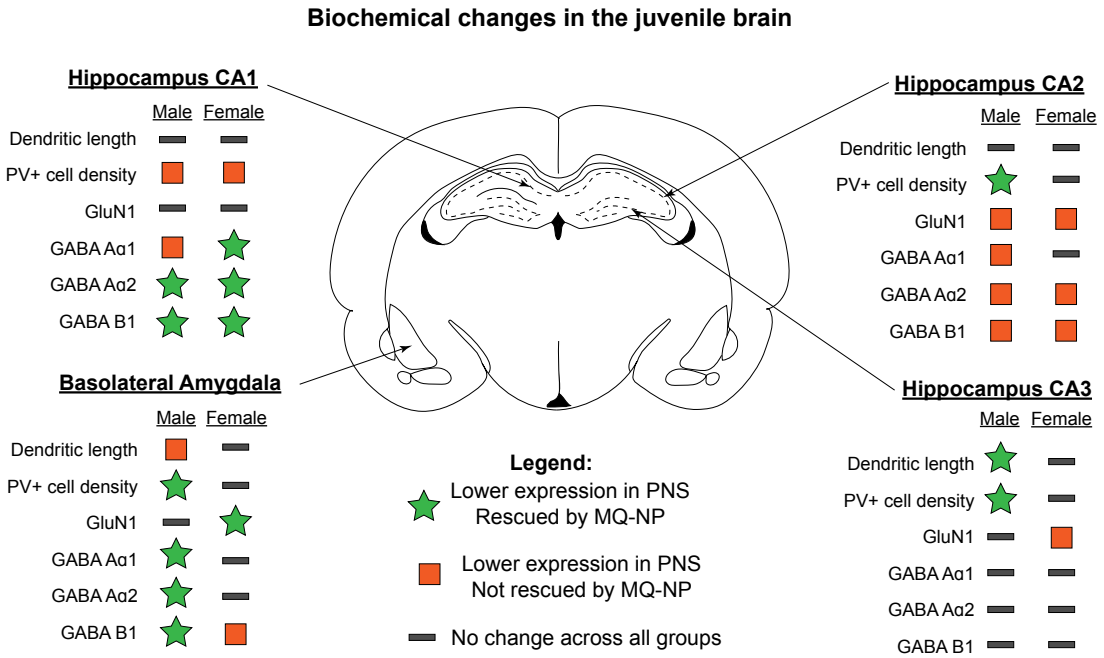


Figure 6.15: Impact of prenatal stress with and without maternal MQ-NP treatment on neurochemical characteristics in the brain of PND30 juvenile offspring. Dendritic length, the number of parvalbumin positive cells, GABA and GluN1 receptor expression were determined by immunohistochemistry**.** Green stars and orange boxes represent decreased expression in the PNS condition. Green stars also represent a normalisation of the expression of these markers with MitoQ-NP administration, while orange boxes represent no rescue observed. Grey dashes represent no differences between control and PNS groups. PNS: Prenatal stress, Veh: Maternal vehicle treatment, MQ-NP: Maternal MitoQ-NP treatment, PV+: parvalbumin positive. Graphs with statistical analyses are presented in Appendix D.

6.3.10 PNS and maternal MitoQ-NP treatment altered effects of placental-conditioned media and foetal plasma when exposed to neuronal cultures

The next aim was to determine if placental secretions play a direct role in foetal programming. Placental-conditioned media was collected and applied to E18 neuronal cultures. The same procedure was also carried out using foetal plasma. A summary of the results are presented in Fig 6.16 and graphs with statistics output are included in Appendix D.

The dendritic length of neurones was shorter, and GluN1, $GABA_{A\alpha1}$, $GABA_{A\alpha2}$, and $GABA_{B1}$ expression was lower in cortical neurone cultures exposed to placental-conditioned media from stressed dams, compared to those exposed to placental-conditioned media from control dams. These changes were not observed when placental-conditioned medium from stressed dams treated with MitoQ-NP was applied to the neuronal cultures, except in the case of $GABA_{B1}$ which was still observed to be lower than controls. The data indicate that maternal MitoQ-NP treatment resulted in the placenta secreting factors that could abrogate the stress-induced changes in dendritic length, GluN1, $GABA_{A\alpha1}$, $GABA_{A\alpha2}$, but not $GABA_{B1}$.

When plasma from stressed foetuses were applied to the neuronal cultures, the observations of decreased dendritic length, lower GluN1, $GABA_{A\alpha1}$, $GABA_{A\alpha2}$, and $GABA_{B1}$ expression were also observed when compared to neurones exposed to plasma from control foetuses. This corresponded with the patterns observed when conditioned media from stressed placenta were applied. However, foetal plasma from the stress/MitoQ-NP group could only prevent the changes in dendritic length, GluN1 and $GABA_{A\alpha2}$ expression in the neuronal cultures, but not the changes in $GABA_{A\alpha1}$ and $GABA_{B1}$ expression.

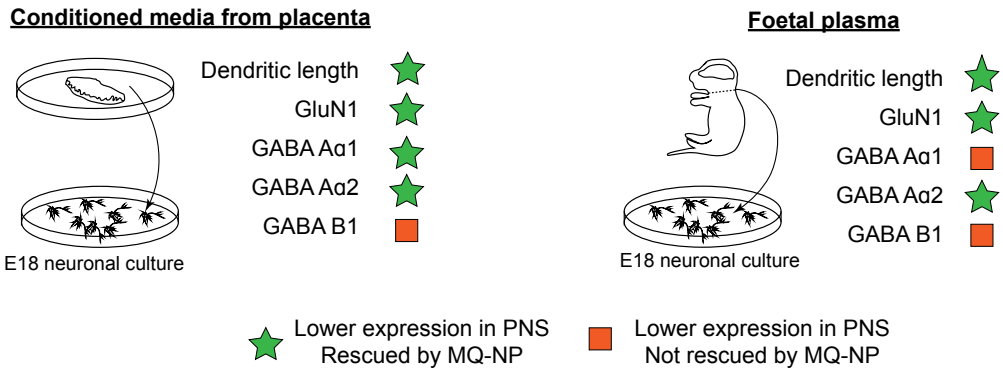


Figure 6.16: Changes in neuronal characteristics of E18 cortical cultures when exposed to conditioned media from placenta or foetal plasma. Green stars and orange boxes represent decreased expression in the PNS condition. Green stars also represent a normalisation of the expression of these markers with MitoQ-NP administration, while orange boxes represent no rescue observed. PNS: Prenatal stress, Veh: Maternal vehicle treatment, MQ-NP: Maternal MitoQ-NP treatment, PV+: parvalbumin positive. Graphs with statistical analyses are presented in Appendix E.

6.3.11 Maternal MitoQ-NP administration resulted in altered 11β-HSD2 gene expression in the placenta

11β-HSD2 mRNA-expressing positive cells in the placenta JZ were also quantified. There was a stress x MitoQ-NP administration interaction in both male ($F_{1,24}$=6.14, p=0.021, Fig 6.17A) and female placenta ($F_{1,24}$=5.93, p=0.023, Fig 6.17B). No main effects of stress nor MitoQ-NP administration were seen.

Stressed placenta tended to show greater number of 11β-HSD2 positive cells compared to controls, in both males (p=0.185) and females (p=0.094). MitoQ-NP treatment in control dams significantly increased 11β-HSD2 positive cell counts in the placental JZ in the males (p=0.018), but not in the females (p=0.244). In the males, placenta from stressed/MitoQ-NP dams had a significantly lower 11β-HSD2 gene expression compared to placenta from control/MitoQ-NP dams (p=0.043). In females, placenta from stressed/MitoQ-NP dams had significantly lower 11β-HSD2 gene expression compared to placenta from stressed vehicle-treated dams (p=0.034). In both sexes, stressed/MitoQ-NP groups did not show differences in 11β-HSD2 positive cell counts compared with the control/vehicle group.

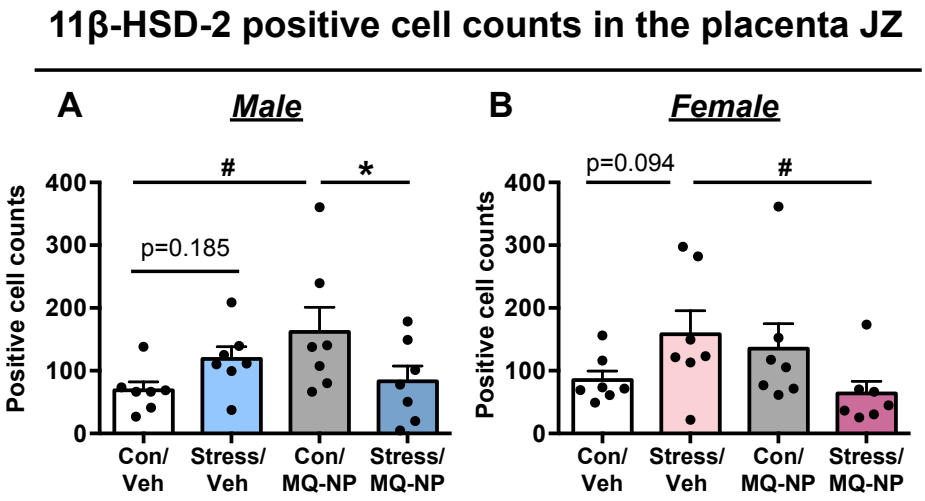


Figure 6.17: Changes in 11β-HSD2 positive cell count in the placenta. (A) Males (B) females. Asterisks represent significant differences between control and PNS groups (where * p<0.05), while hashes represent differences between vehicle and MitoQ-NP treated groups (where # p<0.05). Con: Control, Veh: Maternal vehicle treatment, MQ-NP: Maternal MitoQ-NP treatment.

6.3.12 Maternal stress and MitoQ-NP administration alters circulating steroid concentrations

Plasma neuroactive concentrations were measured using LC-MS, in order to determine if there were any unexpected effects of MitoQ-NP administration in the dam, and/or if there was any cross-talk between the neuroendocrine and cellular stress systems following gestational stress.

As expected, corticosterone concentrations in the maternal plasma was affected by stress ($F_{1,24}$=33.8, p<0.001), whilst the main effect of drug treatment was not significant ($F_{1,24}$=3.11, p=0.09; Fig 6.18A). Stressed groups had greater corticosterone concentrations as compared to the control groups, in line with the trend observed in Figure 6.6 where RIA was used to measure corticosterone. For 11-DHC, there was both a main effect of stress ($F_{1,24}$=19.6, p<0.001) and a main effect of drug treatment ($F_{1,24}$=6.30, p=0.019), but no stress x treatment interactions were observed (Fig 6.18B). Post-hoc testing showed that stressed groups had greater 11-DHC concentrations as compared to the control groups, and additionally in the control groups, MitoQ-NP treatment also resulted in an increase in plasma 11-DHC concentrations.

A main effect of stress ($F_{1,24}$=11.4, p=0.0023; Fig 6.18C), but no effect of treatment, was observed for maternal plasma DOC concentrations. Post-hoc testing showed DOC concentrations were significantly increased following stress only in the MitoQ-NP treated groups (Fig 6.18C), but not the vehicle-treated groups (p=0.152). There was no effect of stress or treatment for plasma DHDOC (Fig 6.18D) and THDOC (Fig 6.18E) concentrations.

The two-way ANOVA also revealed a main effect of MitoQ-NP treatment that was close to significance for maternal plasma progesterone ($F_{1,24}$=3.70, p=0.066, Fig 6.18F), DHP ($F_{1,24}$=3.39, p=0.080, Fig 6.18G) and allopregnanolone ($F_{1,24}$=3.61, p=0.07, Fig 6.18H) concentrations. Post-hoc SNK pairwise comparisons revealed lower levels of these steroids in the PNS/MitoQ-NP groups compared with the PNS/Veh groups, and the difference was significant for allopregnanolone (p=0.029) but close to significance for progesterone (p=0.062) and DHP (p=0.054). For all three analytes, it seemed that plasma concentrations were dramatically decreased with stress and MitoQ-NP treatment but only in four out of the seven dams in the group.

For plasma pregnenolone, there was a main effect of stress that was close to significance ($F_{1,24}$=3.94, p=0.058, Fig 6.18I), however, pairwise comparisons did not reveal any significant differences when individual groups were compared. Lastly, a stress x MitoQ-NP treatment interaction was observed for maternal plasma testosterone concentrations ($F_{1,24}$=5.99, p=0.0022, Fig 6.18J). MitoQ-NP treated control groups had significantly greater plasma testosterone concentrations than the vehicle-treated control group (p=0.0165) and the MitoQ-NP treated stressed group (p=0.0051).

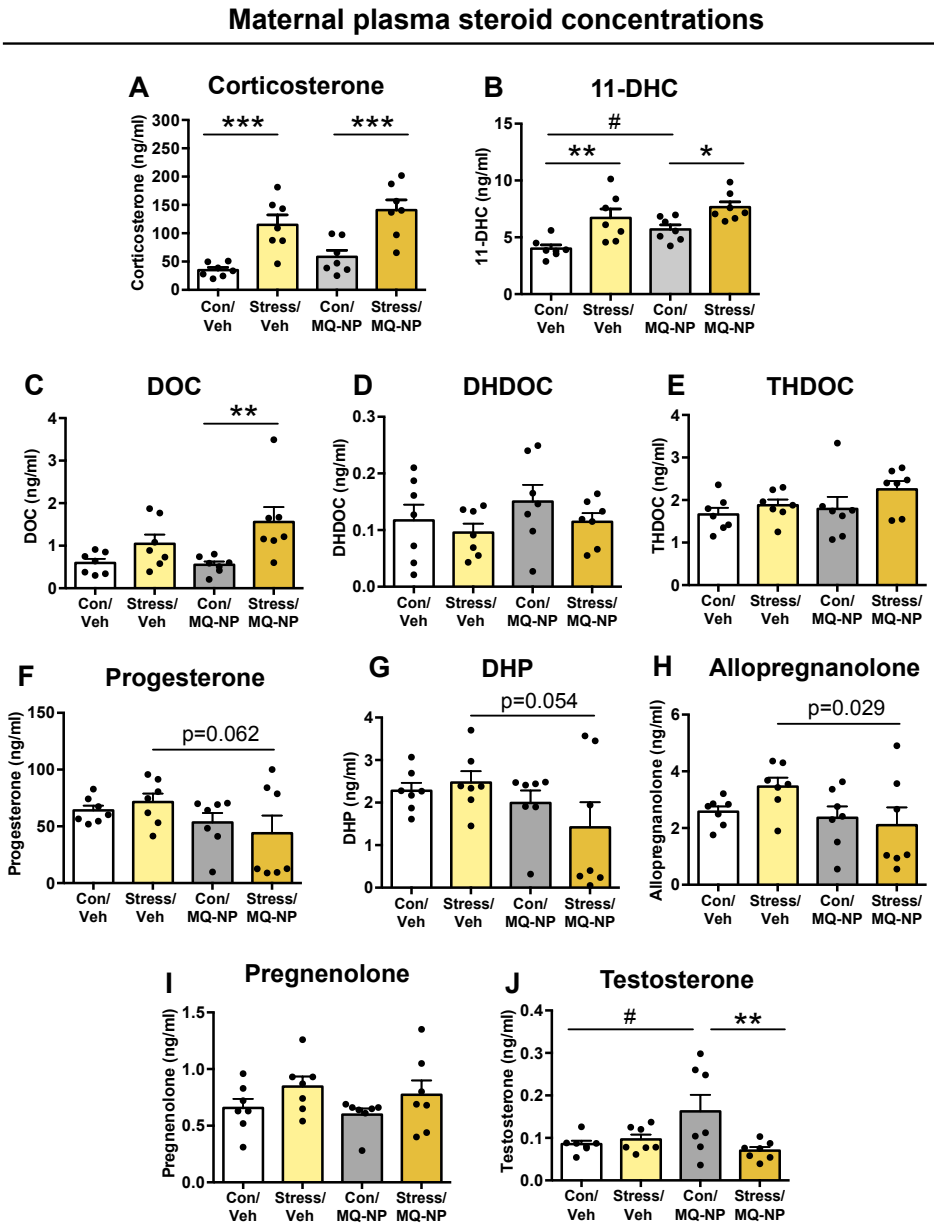


Figure 6.18: Changes in the maternal plasma steroid concentrations, measured by LC-MS. Asterisks represent differences between control and PNS groups (where * p<0.05, ** p<0.01, *** p<0.001), while hashes represent differences between vehicle and MitoQ-NP treated groups (where # p<0.05). Con: Control, Veh: Maternal vehicle treatment, MQ-NP: Maternal MitoQ-NP treatment. n=7 per group.

6.3.13 Summary of results

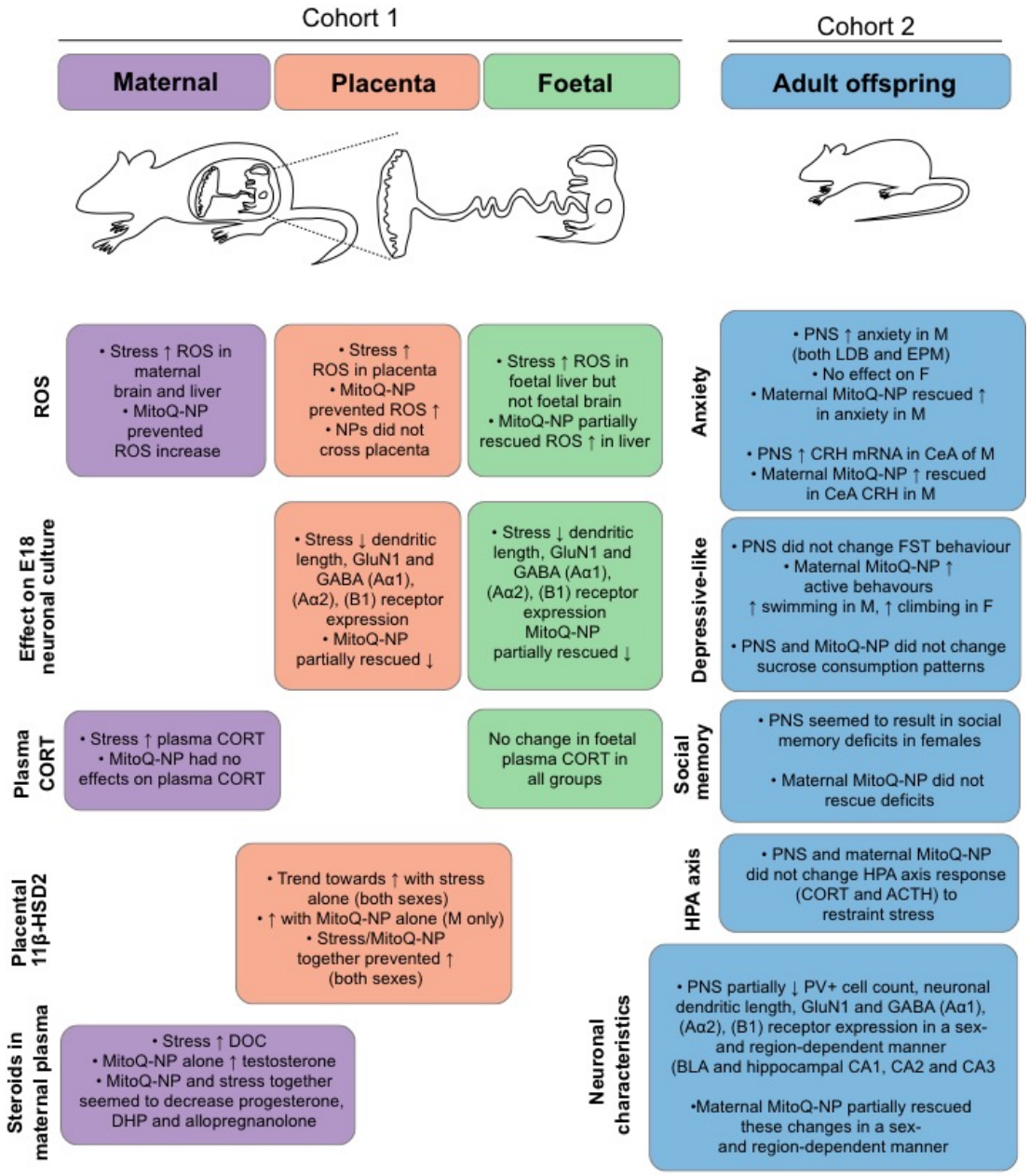


Figure 6.19: Summary of results obtained in this study. Results obtained from the maternal component are presented in purple, the results obtained from the placenta in orange and the foetus in green. In the adult offspring, the results from the behavioural characterisations and other neuronal characteristics are summarised in the right-hand panel in blue. (↑) indicates an increase, (↓) indicates a decrease, M: Males, F: Females

6.4 DISCUSSION

Results of this study are summarised in Figure 6.19. In summary, this study first showed that maternal social stress was associated with increased pro-oxidant production (in the form of ROS) in the maternal brain, liver and placenta. This conclusion was further supported by the use of a maternally targeted antioxidant, which prevented the increase of ROS in these three regions investigated. Stress-induced deficits in behaviour and alterations in neurobiological characteristics of the adult PNS offspring were partially or completely prevented with maternal antioxidant treatment, indicating that maternal/placental ROS play a role in contributing to these negative outcomes in the offspring.

The study also investigated *how* oxidative stress may participate in foetal programming, and the role of the placenta was examined. The results suggest that the stressed placenta secretes "damaging factors" into the foetal circulation, and these "damaging factors" could alter neuronal characteristics (i.e. dendritic length, GluN1 and $GABA_A$ and $GABA_B$ receptor subunit expression) of *in vitro* neuronal cultures. Neutralisation of maternal/placental ROS using MitoQ-NP could reduce the effects of these "damaging factors", and several PNS-induced alterations in neuronal characteristics were completely prevented. These patterns of deficits (with stress) and normalisation (with maternal MitoQ-NP administration) of neuronal markers seemed to mimic that observed in the juvenile offspring brain. It was therefore postulated that foetal programming is possibly a result of the action of these "damaging factors" on neurones during foetal development. Together, these observations add to the list of mechanisms that are known to contribute to the transmission of stress signals from mother to foetus, thereby leading to foetal programming.

Lastly, this study extends the possible use of MitoQ-NP in countering pregnancy-related disorders, in addition to that of hypoxia and pre-eclampsic pregnancies as reported in previous experiments on rats and placental explants (Phillips et al, 2017, Aljunaidy et al., 2018, Scott et al., 2018). It is a proof-of-concept that interventions during pregnancy which target maternal oxidative stress have the potential to prevent negative offspring outcomes associated with prenatal social stress.

6.4.1 Gestational social stress is associated with an increase in oxidative stress

Maternal and placental oxidative stress:

In line with our hypothesis, following psychosocial stress in the pregnant dams, there was an increase in ROS levels in the maternal brain, liver and placenta, implying a greater oxidative stress status in the maternal compartment and in the placenta. This association between psychosocial stress and increased maternal pro-oxidant levels is unsurprising, as it has been shown in both rat and human studies that psychosocial stress can lead to increased oxidative stress in the liver (Ishtiaq et al., 2018) and brain (Schiavone et al., 2013). In another social defeat model, socially defeated male rats also show increased inflammatory markers and decreased antioxidant defences in the brain (Patki et al., 2013). Conversely, preventing the increase of ROS with antioxidant treatment in rats can reduce impairments associated with social defeat (Solanki et al., 2017). The present study showed for the first time that psychological stress during pregnancy in the rat results in increased ROS not only in the brain and liver, but also in the placenta. This also corresponds to observations in human pregnancies, where maternal psychosocial lifetime stress can contribute to placental mitochondrial malfunctioning and the decrease in mitochondrial DNA copy number, conditions which are found associated with increased mitochondrial oxidative stress (Brunst et al., 2017).

Psychosocial stress results in the activation of multiple pathways, and whilst not all of the pathways are investigated here, it was observed that social stress resulted in greater plasma corticosterone concentrations in the female dams (as expected, see section 5.3.1). The increase in ROS production observed in the maternal brain, liver and placenta could potentially be attributed to a downstream effect of increased glucocorticoids, as it is known that HPA axis activation can result in ROS production, due to increased mitochondrial respiration and oxidative phosphorylation (Spiers et al., 2014, Du et al., 2009). *In vitro* studies also showed that the activation of the cytosolic GR by glucocorticoids can trigger downstream molecular pathways such as nitric oxide production (Flaherty et al., 2017) or NADPH oxidase activation (Seo et al., 2012), all of which may contribute to the production of pro-oxidant species. In a gestational corticosterone overexposure model where corticosterone was administered to pregnant mouse dams on E12.5 (and the placenta collected on E14.5), although decreased placental H_2O_2 concentration was found, an increase in

protein carbonylation and glycation, which are cellular outcomes of oxidative stress, was observed in the placentas of female foetuses (Bartho et al., 2019). Undoubtedly, psychosocial stress is accompanied by the production of a barrage of endocrine factors both within the HPA axis (e.g. ACTH, CRH, POMC, vasopressin, melanocyte-stimulating hormone (MSH) etc.) and in the sympatho-adrenal system (e.g. catecholamines like noradrenaline and adrenaline), but also inflammatory cytokines (e.g. tumour necrosis factor α (TNF-α), interleukins like IL-1 and IL-6 etc) (Manoli et al., 2007). All these mediators are involved in signalling pathways in the mitochondria, affecting mitochondrial biogenesis and metabolism (Manoli et al., 2007), and may all interact in a complex manner which can result in different cellular outcomes.

Foetal oxidative stress status:

ROS production was also quantified in the foetal compartment. Whilst prenatal stress was reported to be associated with increased oxidative stress in the rat offspring brain, many of these studies examined offspring during adulthood or adolescence (Zhu et al., 2004, Glombik et al., 2015, Song et al., 2009) but not during foetal stages, therefore does not show the role of ROS in foetal programming during pregnancy.

Here, maternal stress did not result in elevated pro-oxidant levels in the foetal brain at GD20, but an elevation was observed in the foetal liver of stressed dams. This difference in oxidative stress status between the foetal brain and liver was also observed in a previous study by Phillips et al., 2017, where the foetal brain also seemed to be protected from the effects of hypoxia. In another model of prenatal stress in rats (ischemia-reperfusion), the foetal brain was better protected from oxidative stress as compared to other tissues like the heart and muscle following the stressor (Fantel et al., 1998). Together, these data suggest that the development of aberrant behaviours later in life is probably not associated with any changes in the oxidative stress status of the foetal brain *in utero*. This also underscores the need to limit antioxidant treatment to just the maternal compartment, akin to what was done in this study. Whether or not the increase in ROS is directly associated with increased glucocorticoids, which was found to be similarly elevated in the foetal liver but not in the foetal brain in the previous chapter (section 5.3.1), remains to be investigated.

The implications of increased ROS levels in the foetal liver is also not known, but could presumably lead to negative effects in the growth and development of the liver, especially since the period before birth also represents a period where the endogenous antioxidant defence of the foetal liver is at its lowest (Gonzalez et al., 1995).

Pro-oxidant and antioxidant balance:

There are a few other potential issues worth noting in the analysis of oxidative stress status here. Apart from using the DCF assay, other additional tests which measure ROS-induced modifications could have been examined, such as the end products of lipid peroxidation (e.g. malondialdehyde or 4-hydroxynonenal), DNA oxidation (e.g. 8-hydroxy-2'-deoxyguanosine for DNA damage) or protein carbonylation for instance (Frijhoff et al., 2015).

Additionally, although increased ROS is likely to imply higher oxidative stress, one can recall from the introduction that “oxidative stress” is defined as the imbalance between pro- and antioxidant levels, where pro-oxidant activity in a cell is greater than that of antioxidants. As endogenous antioxidant defences were not simultaneously measured in these experiments, it is possible that psychosocial stress may have also upregulated some endogenous antioxidant defences, such that there is no overall increase in oxidative stress in these pregnant rats. However, several other studies in rats have reported quite the contrary, and instead showed that chronic stress and glucocorticoids generally lead to a decrease in the production and activity of antioxidant enzymes like superoxide dismutase (SOD), catalase and glutathione peroxidase, thereby further tipping the balance between pro-oxidants and antioxidants (Zafir and Banu, 2009, Sato et al., 2010).

Sex differences in oxidative stress status:

Additionally, sex differences were not taken into consideration in the analysis of oxidative stress status. In sheep studies, not only did endogenous antioxidant defences differ between different foetal tissues (liver, muscle, brain, kidneys and lungs), there was a sex difference in antioxidant enzymatic activity as well as lipid peroxidation levels, where females appeared to have lower antioxidant enzyme activities, and were therefore considered more at risk of oxidative injury *in utero* in the face of environmental stressors (Al-Gubory and Garrel, 2016). In human studies,

sex-specific alterations were observed in the pro-oxidant versus antioxidant balance in preterm placentae in response to antenatal betamethasone exposure, and male placentae had higher pro-oxidant indices as compared to females (Stark et al., 2011). Whilst male placentae were reported to have higher antioxidant defences in normal human pregnancies, the antioxidant protection was lost in the case of compromised pregnancies, such as in maternal obesity (Evans and Myatt, 2017). Chronic hypoxia also resulted in higher expression of heat shock protein (HSP) 70 and HSP27 levels in female placentae as compared to males in mice, indicating greater oxidative stress in females (Matheson et al., 2016).

Together, these studies indicate the presence of sex differences in oxidative stress status, both in normal and compromised pregnancies, although there did not seem like there was a consistent pattern on which sex tended to be more susceptible or more protected. Given that sex differences in behaviour and neuroendocrine outputs are consistently observed in PNS offspring of this social stress model, it would be neccessary to determine if these differences can be correlated to different oxidative stress levels in the placenta or foetal tissues during foetal development in future studies.

6.4.2 MitoQ-NP was effective in preventing the increase in oxidative stress

MitoQ-NP administration was effective in preventing the increase in ROS production following stress, in the maternal liver, brain and placenta. Stressed dams that were treated with MitoQ-NP had ROS levels that were similar to controls. It is thus possible to conclude that given MitoQ-NP's efficacy in preventing the increase in ROS levels in stressed dams, any differences between MitoQ-NP/stressed groups and saline-treated/stressed groups can be attributed to the normalisation of maternal ROS production.

Since the tissues were collected on GD20, five days after the single dose of MitoQ-NP injection on GD16, MitoQ-NP can be inferred to be efficacious in preventing the ROS increase throughout the duration of the chronic stressor. Whilst previous *in vitro* studies showed that there was a sustained release of MitoQ over 24 hours from the NPs (Phillips et al., 2017), it is not known if its efficacy here can be attributed to a similar sustained release of MitoQ over the five day period, or if there were other permanent changes in the physiology of the pregnant dam.

Apart from effects on the maternal and placenta tissue, maternal MitoQ-NP administration also seemed to result in a modest inhibition of ROS production in the stressed foetal liver, but ROS levels were not completely normalised to control levels. Although it has been shown that NPs are sequestered in the maternal tissues and do not cross the placenta (Phillips et al., 2017), it is not known whether MitoQ itself can be released into to the foetal circulation. However, it has been previously reported that MitoQ uptake is extremely low in the foetal compartment when it was administered to pregnant rat dams via drinking water, even without the presence of NPs (Nuzzo et al., 2018). Moreover, in the hypoxia model, maternal MitoQ-NP did not rescue the ROS increase in the stressed foetal liver (Phillips et al., 2017). Similarly, there was no effect of MitoQ-NP administration on ROS levels in the foetal brain, as observed in this study. These data suggest that the effect of MitoQ-NP on the foetal compartment could be indirect and probably not due to a direct crossover of MitoQ.

6.4.3 Maternal MitoQ-NP administration prevented anxiety behaviour in PNS male offspring

Behavioural analysis:

Maternal MitoQ-NP administration prevented the anxiety phenotype of PNS male offspring in adulthood, and this was supported by data from both the light-dark box and the elevated plus maze. It is known that females and males can show differences in anxiety-like behaviour (Zimmerberg and Farley, 1993, Johnston and File, 1991), however, no differences in baseline exploratory or anxiety-like behaviour was observed in this study. Whilst a sex difference was not observed in the control animals at baseline, unlike the male PNS offspring, female PNS offspring did not show anxiety-like behaviour, and the performance was largely unaltered by maternal MitoQ-NP administration. The finding that PNS females do not show anxiety phenotype is in line with findings from previous studies, and it has been previously reported that anxiety-like behaviour in the females was related to oestrous cycle stage instead of prenatal stress status, with females at pro-oestrous and oestrous stages showing less anxiety-like behaviour (Brunton et al., 2015, Brunton and Russell, 2010, Marcondes et al., 2001). Both the LDB and EPM test for approach-avoidance behaviours, which are ethologically relevant, as these behaviours are based on the rodent's innate conflict between the drive to explore novel environments and aversion to brightly lit open spaces (Rodgers, 1997). Moreover,

deficits in behaviour in both tests have been consistently replicated in PNS male offspring using the social stress model (here, and in Brunton and Russell, 2010, Brunton et al., 2015), indicating that this sex-dependent anxious phenotype is a robust outcome of prenatal social stress.

Whilst the LDB and EPM are similar, the underlying neurobiology between two tests are different and can yield different results in response to different anxiolytic agents (Cryan and Sweeney, 2011). However, the fact that MitoQ-NP could rescue the robust anxious phenotype in both tests provides strong evidence that it is a valuable intervention to potentially prevent developmentally programmed anxiety disorders in the offspring. This is not the first time a maternal antioxidant treatment has been shown to block programmed anxiety behaviour in offspring, as a previous study showed that the antioxidant TEMPOL could also prevent glucocorticoid-induced anxiety when administered to pregnant mouse dams (Roghair et al., 2011). Notwithstanding, the value of MitoQ-NP treatment here, lies in that the antioxidant effects were targeted at the maternal mitochondria, and is unlikely to have any direct effects on the foetus. It is however, worthwhile to note that MitoQ-NP cannot be considered an anxiolytic *per se* as it was administered to the mother and not the offspring themselves in this study.

Apart from the measures related to the approach drive and aversion drive (i.e. measures associated with activity in the lit or open areas), indices of general activity in the test environment were also analysed in this study. In the LDB, PNS males travelled shorter total distances, and this was not observed in the PNS group with maternal MitoQ-NP administration, indicating a normalisation of such behaviour. In the EPM, the total number of entries PNS female rats made into both arms was lower than in controls, indicating lower exploratory behaviour. Again, this behaviour was normalised in the female PNS/MitoQ-NP group. Whilst these two indices may represent a drop in general locomotor activity, a possible confounding factor for these tests, they may also represent altered emotional states (Fraser et al., 2010). These observations could thus also be interpreted as PNS offspring (both males and females) showing more negative emotional states, resulting in decreased exploration of a novel environment in general. If so, these data would further support the contention that maternal MitoQ-NP treatment may be valuable in preventing abnormal affect-related behaviours following prenatal stress.

Anxiety-related changes in the brain:

In support of the behavioural observations, *in situ* hybridisation studies showed that the greater CRH mRNA expression in the CeA usually observed in PNS males was also prevented by maternal administration of MitoQ-NP, both in juvenile rats and adult rats. As CRH overexpression in transgenic mice is associated with anxious behaviour (Stenzel-Poore et al., 1994, van Gaalen et al., 2002), this observation could at least partly underlie the behavioural changes in the LDB and EPM. This is the first time an increase in CRH mRNA expression in the central amygdala (CeA) was observed in juvenile male PNS offspring, as previous findings were all from adult rats. Apart from directly mediating adult anxiety behaviour, elevated levels of CRH were also previously found to be associated with poorer dendritic branching in the developing rat cortex, which also extend into adulthood (Curran et al., 2017). Here, dendritic length seemed to be generally reduced in various brain regions in the PNS juvenile rat brain (Fig 6.16). Whether or not a similar decrease in dendritic length also occurred in the CeA, where increased CRH gene expression was observed, remains to be investigated.

This study also investigated other mechanisms apart from amygdala CRH which may explain the observed anxious behaviour. Anxiety can result from dysregulation of the inhibitory neural circuits that control communication between the different limbic regions (Nuss, 2015). In particular, deficient inhibitory tone in the amygdala, arising from the imbalance in signalling activities of GABA and glutamate for instance, can lead to anxiety-like behaviours (reviewed in (Quirk and Gehlert, 2003)). Blocking $GABA_A$ receptors in the BLA tends to increase anxious behaviours, while enhancing $GABA_A$ function can attenuate these behaviours (Quirk and Gehlert, 2003, Sanders and Shekhar, 1995). In KO mice models, deletion of $GABA_{A\alpha1}$ subunit in the amygdala and BNST results in heightened anxiety on the EPM and open field test (Gafford et al., 2012) and $GABA_{B1}$ KO mice also exhibited increased anxiety in the LDB (Mombereau et al., 2004).

In this study, alterations in $GABA_A$ and $GABA_B$ receptor subunit expression occurred with prenatal stress. $GABA_{A\alpha1}$ and $GABA_{A\alpha2}$ receptor subunit protein expression were lower in the BLA of the PNS males, and was normalised by maternal MitoQ-NP treatment. This observation, which was not present in female PNS offspring, correlates with the anxious behaviour observed in the LDB and EPM, which was

also only present in male PNS offspring but not the females. Nonetheless, as only two $GABA_A$ receptor isoforms were investigated in this study, it is not possible to fully determine how exactly GABA neurotransmission is altered in the PNS rats, due to their pentameric nature and that the arrangement of subunits can also heavily influence their electrophysiological characteristics (Sigel and Steinmann, 2012). Nevertheless, the presence of these changes allude to the fact that GABAergic neurotransmission may be altered in some way in the PNS offspring, and maternal oxidative stress could play a role in altering the normal development of GABA neurotransmitter circuits.

Given that the intervention here was targeted at the pregnant mother during gestation, the above changes in amygdala CRH and GABA receptor subunit expression may already be present during foetal or earlier postnatal development. Previous studies have shown that inducing CRH overexpression in the forebrain and several limbic regions of transgenic mice during early life can contribute to increased anxious behaviour in adulthood (Kolber et al., 2010), whilst prenatal stress induced-changes in various sub-regions of amygdala have been found in neonatal brains as early as PND7 (Kraszpulski et al., 2006), further supporting the possibility that detrimental changes might already have occurred before PND30. During early postnatal development in the rat, $GABA_{A\alpha1}$ and $GABA_{A\alpha2}$ subunit expression patterns also undergo a switch, which possibly provides a window of vulnerability for detrimental changes to occur (Davis et al., 2000). A future line of work could therefore be to examine the foetal (or neonatal) amygdala for the same neuronal markers, to establish if these changes present at even earlier stages in the offspring's life.

Taken together, the behavioural and corresponding immunohistochemical data suggest that maternal oxidative stress during pregnancy contributes to programming of an anxious phenotype in the offspring, and that maternal MitoQ-NP treatment is a viable means of preventing the development of an anxious phenotype.

6.4.4 Prenatal stress did not result in depressive-like behaviour

Apart from anxiety, depression is also one of the major psychopathologies that occur as a result of early life stress (Weinstock, 2017). Depression is a complex psychological disorder that is multi-faceted in humans, but certain features such as anhedonia and helplessness can be tested in rodent models. Two tests were used

in this study to assess whether prenatal social stress results in “depressive-like behaviour” in the adult offspring: (i) sucrose preference test for anhedonia, and (ii) the forced swim test, which is thought to represent behavioural despair.

Sucrose preference test

In the sucrose preference test, there were no significant differences between the total amount of sucrose consumed in the control and PNS rats, for both males and females, which may at first glance, indicate that PNS rats do not exhibit anhedonia. However, a closer look into the individual consumption patterns revealed that PNS females seemed to consume more sucrose than the control females, and this pattern was not observed in the males. There also exists additional confounds related to the sucrose preference test (Strekalova et al., 2011) that may offer various interpretations of these results.

Firstly, the higher consumption of sucrose during the test, as the PNS female rats here tended to show, could represent a sucrose-binging phenotype, which is known to be affected by dysregulation in the HPA axis (Calvez and Timofeeva, 2016), and can be affected by sex and testosterone exposure (Culbert et al., 2018). Additionally, rats need to be singly housed for the test, which might have affected the sucrose drinking patterns, as social isolation is a known stressor that can also result in HPA axis disruptions (Hawkley et al., 2012). The tendency for PNS females to binge on sucrose could therefore have been an outcome of prenatal stress, but may also be driven by a different susceptibility (from males) to the inevitable isolation stress brought about by the test paradigm. Indeed, protracted social isolation has been shown to increase the amount of sucrose consumed in mice (Coudereau et al., 1999). Furthermore, it was reported that female rats are generally more affected during isolation stress, manifesting as an increased inflammatory response (Hermes et al., 2006) and decreased excitability of PVN CRH neurones (Senst et al., 2016). Although speculative, these changes may interact and manifest as differences in sucrose consumption patterns between males and females.

Additionally, there seemed to be a sex difference at baseline in terms of how control males and females performed in the sucrose preference test. While all but one control male preferred sucrose from the first day, in the control females, it seemed like there was a larger difference between the sucrose consumption of female rats on the first and second day of testing. On the first testing day, four out of eight

control female rats preferred water over sucrose, but this was not apparent on the second testing day. Although rats were acclimatised to the testing cage one day prior to the test day, they were still sucrose-naïve and neophobic responses could still have occurred on the first testing day. Whilst a previous rat study showed that prenatal stress increased neophobic responses (Pfister et al., 1981), the reverse was observed here, and it was instead some of the control females, instead of the PNS females, that seemed to exhibit a neophobic behaviour. Nonetheless, the pathways underlying neophobia and variations in sucrose consumption are complicated and have been reported to be controlled by oxytocin signalling pathways (Amico et al., 2005), or the dopaminergic system (Tonissaar et al., 2006), both of which were not investigated here.

In any case, stressed females with maternal MitoQ-NP administration seemed to show a more similar pattern of sucrose consumption as the control females than the PNS females, which suggest that prenatal stress or maternal antioxidant treatment could have interacted and differentially altered these abovementioned pathways. Nonetheless, it is worthwhile to note that the statistical tests were inconclusive here, and this is compounded by the presence of a large inter-individual variability in sucrose consumption patterns, which has been reported to be quite common by other groups as well (Brennan et al., 2001, Tonissaar et al., 2006). Ultimately, given the prevalence of such variability, more rats need to be tested on this paradigm before any definite conclusions can be drawn.

Forced swim test

Prenatal social stress also did not result in changes in behaviour in the forced swim test in both males and females. While prenatal stress is generally suggested to increase FST immobility time in the offspring in several rat studies (Morley-Fletcher et al., 2003, Montes et al., 2016, Alonso et al., 1991), the lack of an effect here could be due to variability induced by differential maternal stress paradigms, as maternal restraint instead of social stress was used for all of these previous studies. Nonetheless, it is worth noting that differences in methodology of the swimming test itself could also contribute to the contradictory results (Bogdanova et al., 2013). For instance, factors related to the experimental animal, such as sex, age, body weight, strain of rat, experience of previous behavioural testing can all affect behaviour in the FST, while subtle modifications in the methodology of the FST itself, such as

water temperature, size of the pool, lighting and duration of swim can all markedly affect the outcome of the results (Kara et al., 2018).

While males and females were analysed separately here, sex is indeed a factor that can affect behaviour in the FST (Bogdanova et al., 2013, Kokras et al., 2015). There are however, conflicting conclusions in the literature on how FST behaviours may be different, with a third of reviewed studies reporting that females exhibit more immobility, one-third reporting that females present less immobility than males, and a last third reporting that males and females show no significant differences in immobility (reviewed in (Kokras et al., 2015)). Additionally, some studies have also pointed out that pro-oestrous and oestrous female rats had greater immobility, although other studies reported no differences across oestrous stage (Consoli et al., 2005, Bogdanova et al., 2013). In this study, despite PNS not having an effect on FST behaviour, it was observed that maternal MitoQ-NP administration altered FST behaviour in a sex-dependent manner, where an increase in active-coping behaviours was observed, but the styles of active-coping was different for males and females (i.e. increased cimbing in females, and increased swimming in males after maternal MitoQ-NP administration). Additionally, while females did not have different frequency of floating bouts as compared to males at baseline, it was observed that females had greater frequency of climbing bouts compared to males at baseline. This highlights the importance of analysing specific types of behaviours (i.e. floating, climbing and swimming) rather than only investigating immobility time, due to inherent differences in FST performance between males and females.

In terms of the relevance and interpretation of these behaviours, if one were to regard the FST as a test for behavioural despair, then it appears that although prenatal social stress was not “pro-depressant”, maternal MitoQ-NP, remarkably, has “antidepressant effects”. However, it has been proposed in the recent years that instead of being a representation of despair or helplessness, the FST should be interpreted as a measurement of stress coping strategy (Commons et al., 2017, Slattery and Cryan, 2012, de Kloet and Molendijk, 2016). In fact, in the most recent “state-of-the-art” article, it was reported that amongst published work in the three years preceding June 2018, the number of studies interpreting floating behaviour to be “depressive-like” has massively decreased, whilst the number of studies referring to FST as a test of “coping” behaviour has increased (Molendijk and de Kloet, 2019). In light of these shifting trends in the field, it could therefore be proposed that here,

PNS also did not affect the coping strategy of offspring in the FST. As a two-session swimming test was used, which involved a 10 min preconditioning session and a 5 min test 24 hr later, the coping strategy would therefore also encompass an element of learning and memory (de Kloet and Molendijk, 2016, Bogdanova et al., 2013). Hence, it can also be concluded that learning and memory processing following acute swim stress do not seem to be affected in PNS offspring.

Secondly, it is also worth noting that as MitoQ-NP was administered to the pregnant dam and not the offspring, the MitoQ-NP drug itself should not be interpreted as constituting an antidepressant effect *per se*. Instead, it could be contended that maternal antioxidant treatment could have altered neurodevelopmental pathways which regulate stress coping behaviours during adulthood. Regardless, symptoms of depression and the stress response share similar regulatory mediators and circuitries (Gold et al., 1988). The fact that alterations in behaviour exist in the FST following maternal antioxidant administration suggest that these pathways are sensitive to environmental changes (especially that of oxidative stress status) *in utero* and have the propensity to be developmentally programmed.

The observed FST behaviour is therefore probably due to a complex interaction of various factors, which may include disruptions to serotonergic or noradrenergic signalling, to name a few (reviewed in (Cryan et al., 2005)). Although GABA targeting modulators are not often used as antidepressants, GABA neurotransmission can still play a crucial role in modulating behaviours related to depression (Möhler, 2012, Kalueff and Nutt, 2007). Of note, neuroactive steroids can increase mobility in the FST, possibly through enhancing GABA signalling, although the response can also be modulated by serotonergic agents (Khisti and Chopde, 2000). Additionally, blockade of the glutamatergic NMDA receptor has been associated with the production of fast-acting antidepressant responses, albeit in mice (Autry et al., 2011), while changes in NMDA receptor action (e.g. GluN1 and GluN2A) using an NMDA receptor antagonist in the hippocampus and striatum following prenatal stress in rats can also result in increased immobility time in the offspring (Sun et al., 2013). These studies show the involvement of glutamatergic signalling in modulating the behaviour on the FST as well. In this study, although several receptor expression studies of GluN1, $GABA_A$ and $GABA_B$ have been carried out, there was no single marker with expression patterns explicitly different

between maternal MitoQ-NP treated groups and vehicle groups, suggesting that the observed behaviour may not be attributed to receptor expression differences.

Concluding this section, firstly, given the concerns surrounding the interpretations of these two tests, one must be prudent in analysing the observed behaviour, and not hastily draw conclusions on whether the PNS model here is “pro-depressant” or if a drug is an “antidepressant”, or to ascribe the behaviour as advantageous or disadvantageous to an organism. In any case, regardless of these interpretations, the fact that the PNS offspring did not differ in the behaviour to the controls indicate that the FST might not be a robust behavioural phenotyping test for the social stress model. Nonetheless, the observation that maternal MitoQ-NP influenced offspring behaviour adds weight to the idea that the development of neuronal circuits regulating affect/stress coping during development are vulnerable to maternal environmental changes *in utero*, especially changes associated with oxidative stress.

6.4.5 Maternal MitoQ-NP did not affect social memory performance in female offspring

There seemed to be an impairment of olfactory social memory in the PNS female offspring, although statistics did not reach significance. The average investigation time during the exposure phase and during the test phase were largely similar to those reported in previous studies using the same paradigm (Grundwald et al., 2016) but a larger variability seemed to be present in this study. The variability in the data could have been attributed to the lack of consideration of oestrous cycle stages in the females, which has been shown to affect social recognition memory in mice (Sanchez-Andrade and Kendrick, 2011). Although the use of an inanimate bead instead of a live juvenile rat is a modification from the original social recognition test (Thor and Holloway, 1982), the inanimateness of the object is unlikely to cause any problems, as previous studies have showed that this method of using beads impregnated with odour has been effective in substituting for a juvenile conspecific (Grundwald et al., 2016). It may in fact even be advantageous to use an inanimate object impregnated with odour instead of juvenile conspecifics, as interactions with a live rat can be confounded by anxiety (Starr-Phillips and Beery, 2014).

However, the way in which the odours were coated on the beads in this experiment could have been a problem. It has been previously shown that rats discriminate

individual conspecifics based on their individually distinctive odours, and were able to associate olfactory signatures (i.e. urine, glandular, vaginal or salivary secretions) on objects as representations of these individual conspecifics (Gheusi et al., 1997). Here however, beads were impregnated with odour by being buried in the bedding of cages housing groups of rats, therefore would take up the scent of more than one rat in the cage. If the olfactory system of the rat is indeed adapted towards processing the olfactory cues of individual conspecifics for social recognition, the presence of more than one scent on the bead could have been confusing and might have affected how rats responded and remembered these scents. A similar experiment, but with beads coated with the scent of individual rats should be conducted in the future. Nonetheless, here, maternal MitoQ-NP administration did not seem to improve social memory in female PNS offspring.

The deficits in the social memory in this model have been attributed to decreased V1a vasopressin receptor expression (Grundwald et al., 2016), which was not investigated in this study. It has also been proposed that the hippocampal CA2 region is critical for the formation of social memories (Hitti and Siegelbaum, 2014, Chevaleyre and Piskorowski, 2016). GABA antagonists were also found to alter social memory performance, through increasing excitatory inputs from CA2 to the CA1 (Meira et al., 2018) and lateral septum (Leroy et al., 2018). Here, neuronal and receptor characterisation did not reveal differences in the hippocampal CA2 between controls and PNS in terms of the number of PV+ cells nor dendritic markers, but $GABA_{A\alpha2}$ and $GABA_{B1}$ expression was lower following PNS (Fig 6.15). The lower $GABA_{A\alpha2}$ and $GABA_{B1}$ expression was not normalised in the PNS offspring with maternal MitoQ-NP treatment, which correlated with the observed behaviour. Alterations in $GABA_{A\alpha2}$ and $GABA_{B1}$ expression patterns in the CA2, which were unchanged by maternal MitoQ-NP, could be a possible underlying neurochemical explanation to the observed social memory deficits.

Additionally, it has been suggested that hippocampal NMDA neurotransmission is also important in the regulation of social memory (Gao et al., 2009). NMDA administered subcutaneously before the social memory test in male rats improved performance (Hlinak and Krejci, 2002). Here, whilst GluN1 expression was not different between controls and PNS in the CA1 region, its expression was decreased in both the CA2 and CA3 region and this decrease was not normalised by maternal MitoQ-NP expression. Deletion of GluN1 in the CA3 of the

hippocampus results in mice with decreased social approach (Finlay et al., 2015), while GluN1 knockout in the dentate gyrus of mice results in reduced social memory (Segev et al., 2018). The decrease in GluN1 in certain hippocampal regions in PNS females could therefore be potentially another underlying mechanism of the social memory deficits.

In conclusion, in contrast to the observations in the tests for anxiety and depressive-like behaviour, it does not seem like maternal MitoQ-NP had an effect on performance in the social memory test. This seemed to correlate with decreased expression of several GABA and glutamate receptors in the certain brain regions which are known to be involved in social memory, which were not normalised with maternal MitoQ-NP administration. Nonetheless, this merely describes a co-occurrence and further tests need to be carried out to elucidate the exact role these receptors play in regulating social memory. This also suggests that the programming of socially-related offspring behaviours might not be attributed to maternal oxidative stress. Social memory could very well be affected by other signals *in utero*, or even postnatal maternal care for example, as disruptions in maternal care can result in stark deficits in social memory and social interaction in rats during adulthood (Todeschin et al., 2009).

6.4.6 HPA axis hyperactivity in the offspring was not observed

In this study, prenatal social stress did not result in a hyperactive HPA axis in PNS offspring in this study, for either ACTH or corticosterone concentrations in both males and female rats, which contrasted with previous studies using this model (Brunton and Russell, 2010). Although similar paradigms were used (e.g. restraint stress in adult offspring), these experiments were carried out in different facilities with different cage set-ups, and subtle differences in environmental conditions are likely to have contributed to these discrepancies.

In both cases, blood sampling was carried out in specialised open-top cages which facilitated blood-taking from a venous cathether, and rats were transferred to these cages immediately after the surgery. However, in previous studies carried out in another facility, rats were housed in conventional open-top cages from birth until the surgery, whilst in this study, rats were housed in IVCs. It is known that IVC housing alters the behaviour of mice in the various behavioural tasks, in a strain and sex-dependent manner (Mineur and Crusio, 2009). Housing differences may therefore

be a potential confounding factor which can explain why the previous results using the same social stress model could not be replicated. Additionally, as rats were given an analgesic (carprofen) in this study following cannulation surgery, it is not known this could have interfered with HPA axis responses. Carprofen is a non-steroidal anti-inflammatory drug (NSAID), and given that inflammatory processes can also modulate the stress response, it is unclear if carprofen administration would have confounded the results.

Whilst these potential confounds also reflect one of the conclusions in chapter 4, that the HPA axis is sensitive to many environmental changes, it is worth noting that MitoQ-NP had no effect on HPA axis responsivity either, in both males and females, indicating that the effects of MitoQ-NP are selective, and apply to certain behaviours or physiological parameters, and not the others.

6.4.7 The role of the placenta in the transmission of stress signals from mother to foetus

Thus far, the discussion has been centred on the observations that (i) MitoQ-NP was able to effectively abrogate increased oxidative stress following maternal social stress (sections 6.4.1 and 6.4.2); and (ii) the effect of maternal MitoQ-NP administration on behaviours in offspring (sections 6.4.3 to 6.4.6). Here, the discussion proceeds to unravel how MitoQ-NP may exert its effect, and the focus placed on the placenta – the maternal-foetal interface.

Placental secretion of "damaging factors" into the foetal circulation:

The idea that placenta explants can secrete factors *ex vivo* is not new- in the 1970s, reports showed that active factors present in placental-conditioned media can affect the growth of cells in cultures (Burgess et al., 1977). Here, it was shown that when placental-conditioned media from stressed pregnancies was applied to embryonic cortical neuronal cultures, it caused changes in receptor expression, and these changes were not observed when placental-conditioned media from stressed pregnancies with MitoQ-NP pre-treatment were applied (Fig 6.16). Likewise, similar deficits (with stressed pregnancies) and normalisations (with MitoQ-NP treated stressed pregnancies) were observed when foetal plasma was applied to the neuronal cultures (Fig 6.16). It is therefore suggested that the placental secretions and foetal plasma contained 'factors', both of which can affect neuronal growth in a

similar way. On a side note, although it has been proposed that it was “damaging factors” that were causing this effect (Curtis et al., 2014, Scott, 2018), given the various functions the placenta has, it is likely that apart from an increase of “damaging factors”, the *lack* of “protective factors” resulting from oxidative stress could also contribute to these detrimental changes.

Generally, the directionality of change in the phenotypes of neurones in the E18 cortical cultures when exposed to conditioned media or foetal plasma seemed to correspond to the patterns observed in the juvenile offspring’s brain (comparing Fig 6.15 and Fig 6.16). *In vitro* neuronal cultures have generally been proposed to be good surrogates for *in vivo* neuronal platforms (Belle et al., 2018), and electrophysiological studies show no major differences in the firing patterns between embryonic and adult neurones (Evans et al., 1998). Certain adult patterns of neurone subtype characteristics, such as the 1:5 GABAergic/glutamatergic ratio, are already established during early stages of embryonic development before E14.5 (Sahara et al., 2012). Although these indicate that the *in vitro* embryonic model is valuable, one must note that there are many characteristics between the two experiments that are discordant, thus, caution should be taken when comparing the results of these experiments. Firstly, the neurones *in vitro* are obtained from cortical neurones, whereas in the juvenile rats, brain regions investigated were not cortical in nature (i.e. hippocampus and basolateral amgydala). Secondly, even if the neurones were from the same regions of interest, *in vitro* systems lack the complexity and connections between different sub-regions of the brain. Thirdly, the *in vitro* neuronal cultures were embryonic in nature and did not undergo maturation, unlike those studied *in vivo*. The postnatal period is an important period where dramatic changes in the cellular composition of the brain (e.g. neurone-to-glia ratio) can occur (Bandeira et al., 2009). The GABA neurotransmission network also undergoes crucial reconfigurations that can be affected by neurosteroids for instance (Brown et al., 2016), accompanied by the postnatal switch between $GABA_{A\alpha1}$ and $GABA_{A\alpha2}$ receptor subtypes (Laurie et al., 1992, Fritschy et al., 1994). Postnatal influences, which were not considered here, thus have a great propensity to alter the neuronal characteristics that could have been programmed *in utero*, therefore it may not be conclusive to make the direct association between the *in vivo* juvenile brain and *in vitro* embryonic culture results.

Nonetheless, this *in vitro/ex vivo* study provides strong evidence that the placenta actively secretes factors that can alter neuronal growth and dynamics, and it is through this manner that it may play a role in shaping the pregnancy and contribute to foetal programming (Burton et al., 2016). In order to have a clearer understanding of how these secretions may alter neuronal characteristics *in utero*, the same immunohistochemistry experiments can be carried out in the foetal rat brains. This experimental set-up would allow for a more appropriate comparison between *in vitro* results and *in vivo* results (in the foetal rat brain), as a similar time point could be investigated. An alternative way of investigating the effects of these placental secretions is to inject the conditioned media directly into the brains of postnatal rats (as previously done so in Curtis et al., 2013), then allow the juvenile offspring to develop until PND30, and similar behavioural and biochemical characterisations as that of the PNS juvenile rat brains could be carried out.

The current results also suggest these placental factors may be secreted and released into the foetal circulation, evident from the similar pattern of results from the conditioned media and foetal plasma data (Fig 6.16). However, it should be recognised that there would certainly be differences between the contents of the conditioned media and foetal plasma. Similar to what was proposed in the previous chapter, contents of the foetal plasma would also contain molecules secreted by the foetal tissues, and further metabolism of the contents of the transported placental secretions could have taken place. Furthermore, given that there is a two-way transfer of substances across the placenta, the placental-conditioned media may also contain molecules secreted into the maternal compartment.

At this juncture, one yet unaddressed gap is clearly evident – it is still not known what exactly these “damaging” or “protective” factors in the placenta conditioned media/ foetal plasma are. As mentioned above, metabolomics approaches which can screen for a large number of proteins, amino acids and other compounds, can be used to elucidate differences between normal and compromised pregnancies in clinical studies (Fanos et al., 2012). Previously, placental-conditioned media from hypoxia-exposed dams have been shown to have higher levels of glutamate and tryptophan (Curtis et al., 2014). In the same study, application of glutamate NMDA/AMPA antagonists rescued the decrease in dendritic length observed in cortical cultures, further supporting the idea that increased glutamate could have led to these detrimental morphological changes. Other metabolomic studies using

placental-conditioned media from human placental explants have also identified perturbations in glutamate and tryptophan metabolism e.g. in pre-eclampsic pregnancies (Dunn et al., 2009, Horgan et al., 2010). Tryptophan metabolism, thus, could also be an area of particular interest as it is known that the placenta secretes serotonin, for which tryptophan is a precursor (Bonnin et al., 2011). Placental-derived serotonin was found to be important for foetal brain development, thereby could play a crucial role in foetal programming.

Apart from the secretion of hormones, amino acids and molecules, with the recent boom in epigenetics, it has also been demonstrated that the human placenta can also secrete microRNAs into both the maternal and foetal compartments (Chang et al., 2017). MicroRNAs regulate mRNA expression levels and have been shown to be involved in the pathogenesis of neurodevelopmental disorders (reviewed in (Sun and Shi, 2015)). In the study by Phillips et al. 2017, there was indeed differentially secreted microRNAs between stressed and non-stressed placentae, and some of these differences were abrogated with maternal MitoQ-NP administration. MicroRNAs are secreted into exosomes, which are extracellular vesicles that can cross the blood brain barrier, therefore can exert direct effects on the brain (Scott, 2017). When a transcriptomic analysis of the foetal brains was carried out in Phillips et al., 2017, predicted target genes of those differentially altered microRNAs were also differentially altered in the foetal brain, strongly suggesting that these microRNAs play a direct role in the neuronal development in the foetal brain.

Other changes in the placenta:

Besides being able to affect epigenetic processes in the foetal brain as mentioned above, the placenta itself is also susceptible to epigenetic changes (Maccani and Marsit, 2009). Apart from secreting microRNAs, the placenta itself is also a target of microRNAs (Hayder et al., 2018). Another common epigenetic alteration in the placenta is DNA methylation (Bianco-Miotto et al., 2016), where methylated CpG islands located in gene promoter regions can affect the binding of transcription factors and therefore the expression of target genes. Oxidative stress can alter the methylation of genes in the placenta (reviewed in (Thompson and Al-Hasan, 2012)). The expression of important placental genes such as 11β-HSD2 mRNA are known to be heavily controlled by methylation, where hypomethylation is observed to lead

to an increase in placental 11β-HSD2 mRNA expression in clinical samples (Alikhani-Koopaei et al., 2004).

It was established in the previous chapter that gestational social stress can increase 11β-HSD2 mRNA expression in the placental JZ. Here, surprisingly, there was also a trend towards an increased 11β-HSD2 expression in the junctional zone following MitoQ-NP administration alone. Peculiarly, this was not observed when both stress and the antioxidant were administered. It is not known what could have caused the changes in 11β-HSD2 mRNA expression, but methylation changes in the placenta following MitoQ-NP administration might occur. The increase in 11β-HSD2 expression following MitoQ-NP administration only in the non-stressed condition but not in the stressed condition indicates that the placenta responds differently to disequilibrium in redox balance, and it may be that both ends of the perturbation (too much, or too little ROS) could influence methylation of the 11β-HSD2 gene. It is difficult to ascribe functional significance to these changes, and it is also not possible to determine if these would result in downstream effects in glucocorticoid regulation in the dams, or if it represents a compensatory effect. The small sex difference in male and females, which was also observed in the preceding chapter, is however worth commenting on, as it further supports the contention that male and female placenta can respond differently to the same environmental cues. The administration of folic acid, which has known antioxidant properties, to pregnant rats results in a decrease in 11β-HSD2 mRNA expression in male, but not female placentas (Penailillo et al., 2015). It is likely that there are complex interactions between oxidative stress, placenta sex, and epigenetic mechanisms (for instance, placental OGT, which is X-linked and involved in chromatin regulation and transcriptional control (Bale, 2011) which deserve further investigation. It also hints to the fact that changes to the pro-oxidant/ antioxidant balance may have knock-on effects on other systems.

6.4.8 Other changes in the maternal compartment

Although MitoQ-NP is considered safer in that it does not cross into the foetal compartment, other maternal side effects such as changes to steroid production may still occur, especially since mitochondria are an important site of steroid production (Miller, 2013)(Fig 3.1). In this study, corticosterone production following stress, as a result of HPA axis activation, did not seem to be affected by MitoQ-NP

administration. However, MitoQ-NP administration seemed to alter the plasma concentrations of several steroids differentially in control and stressed dams, suggesting that there is a complex interaction between stress and the antioxidant status in pregnancy.

Additionally, insofar as neuroactive steroids in pregnancy are concerned, most of the studies have focussed on the role of allopregnanolone and little is known about the role of other steroids such as DOC and its metabolites. These results therefore also provide a reference interval for future studies on steroid metabolism in rat pregnancies, and can also shed light on some of the lesser studied steroids that may have an important role to play in mediating the impact of insults during pregnancy.

Testosterone:

Firstly, there was an increase in plasma testosterone concentrations following MitoQ-NP administration in the control groups, but not in the stressed groups. Testosterone has been regarded as a pro-oxidant, at least in the cardiovascular system, and can increase ROS production *in vitro* (Tostes et al., 2016). As ROS is important for normal cellular signalling, too much antioxidants may also disrupt cellular function. Following this argument, there is a possibility that the increase in testosterone levels could have been a compensatory action, perhaps an attempt at reinstating the pro- vs antioxidant balance which was altered by the antioxidant treatment.

The implications of this testosterone increase in control/MitoQ-NP dams are unknown, as it was not determined here if the increased testosterone was being transferred to the foetus. However, increases in plasma testosterone in pregnant rats are often associated with poorer offspring outcomes, leading to offspring anxiety (Hu et al., 2015), IUGR (Sathishkumar et al., 2011), hypertension (Chinnathambi et al., 2014) and altered sexual function (Ramezani Tehrani et al., 2013). Although the control/MitoQ-NP offspring did not show any anxious behaviour in this study, it cannot be ascertained if other disruptions to the control/MitoQ-NP offspring would have occurred.

Progesterone, DHP and allopregnanolone:

A trend towards an increase in plasma allopregnanolone, DOC and pregnenolone in the pregnant dams was observed with social stress alone, and this possibly reflects the acute production of neuroactive steroids after an acute challenge (chapter 4). However, when MitoQ-NP was given prior to chronic social stress, maternal plasma progesterone, DHP and allopregnanolone seemed to decrease instead. A large variation was observed, and extremely low levels of these steroids were particularly obvious in a few rats.

Progesterone and its metabolites is known to have antioxidant and protective properties following insults (Zampieri et al., 2009). However, given their importance in maintaining the pregnancy, it thus seems extremely counterintuitive, or even dangerous, that a lower concentration is observed in the case of gestational stress. However, the relationship between antioxidant status and progesterone production is likely to be complex. The production of progesterone is dependent on the antioxidant enzyme SOD (Sawada and Carlson, 1996), and the administration of a supraphysiological dose of antioxidant (MitoQ in this case) could have affected the body's own antioxidant production (of SOD, for instance) in the corpus luteum, affecting the endogenous production of progesterone. Nonetheless, this is merely a postulation and whilst offspring of stressed/MitoQ-NP treated dams seemed to show a rescue in several aberrant behaviours, it is not known if this slightly lower progesterone, DHP and allopregnanolone could have any other downstream side effects on the mother or the pregnancy.

Oxidative stress and glucocorticoids:

Lastly, although MitoQ-NP did not alter corticosterone concentrations, the cross-talk between the oxidative stress system and glucocorticoid metabolism is stil noteworthy. Whilst it is possible that the stress-induced ROS increase is a downstream effect of corticosterone production, conversely, the glucocorticoid system can also be regulated by changes in oxidative stress status. Increased H_2O_2 negatively regulates GR function *in vitro*, decreasing downstream target gene expression (Makino et al., 1996). Pituitary corticotroph cells with elevated H_2O_2 are also unable to properly regulate negative feedback inhibition *in vitro* (Asaba et al., 2004). Antioxidant treatment in rats on the other hand, can also result in the activation of the HPA axis, through downregulation of GR and MR in the pituitary gland (Prevatto et al., 2017). Therefore, as much as glucocorticoids can result in the

detrimental tipping over of ROS, changing of the redox balance could also affect HPA axis and glucocorticoid function in different ways, and indiscriminate antioxidant administration may bring about unintended effects. For instance, whilst combined glucocorticoid and antioxidant therapy can be safe and beneficial for pregnancy compromises like that of pre-term births, glucocorticoids alone, or antioxidant therapy in healthy infants, do not offer the same beneficial effects (Camm et al., 2011) .

Converging point between steroids and oxidative stress?

Given the seemingly complex interactions between oxidative stress status and steroid production and action, there are likely to be various converging points between these two systems. Of note, the nuclear factor erythroid 2-related factor 2 (Nrf2) signalling pathway seems to be a possible candidate that lies in the intersection between all these processes (Kovac et al., 2015, Li and Kong, 2009).

Nrf2 is a transcription factor that is heavily involved in the antioxidant response (da Costa et al., 2019). In physiological conditions, Nrf2 activity increases in parallel with ROS, where it translocates to the nucleus to activate endogenous antioxidant defences and other protective cellular mechanisms (Tebay et al., 2015). MitoQ is known act through various pathways that involve Nrf2 activity (Zhou et al., 2018, Zhang et al., 2019, Hu et al., 2018). Nrf2 activation is one of the downstream effects of steroid-receptor binding for progesterone (Zhang et al., 2017), whilst Nrf2 knockout models show that the production of teststerone could be affected (Chen et al., 2015). Social defeat in rats also prevents Nrf2 translocation and increases the vulnerability to depression (Bouvier et al., 2017), indicating that Nrf2 may play a role in stress responses as well. Mouse studies have started to explore the links between Nrf2 and pregnancy, where Nrf2 activity is crucial for maintaining maternal hepatic adaptations to pregnancy (Zou et al., 2013).

In terms of interaction of Nrf2 and the glucocorticoid system, a previous study in cell lines reported that increased glucocorticoids was accompanied by a suppression of the Nrf2-dependent antioxidant response (Kratschmar et al., 2012), whereas a more recent study reported that maternal corticosterone treatment to pregnant mice did not in fact alter protein expression of Nrf2 (Bartho et al., 2019). Whilst the involvement of Nrf2 in gestational social stress is only speculative, it could be a

potentially important common target between the neuroendocrine and oxidative stress system during pregnancy that is worth investigating in the future.

While these changes in plasma steroid concentrations are present, it cannot be ascertained if these are compensatory mechanisms to restore disequilibrium in oxidative stress status, or merely a co-occurrence, therefore it is difficult to ascribe functional significance to these changes. However, the presence of these somewhat paradoxical changes attests to the idea that antioxidant therapy may affect numerous other endogenous systems via common cellular signalling pathways.

6.4.9 Limitations of MitoQ-NP and implications of MitoQ-NP as a potential therapeutic intervention for stress-induced prenatal programming

Whilst MitoQ treatment has been widely accepted to be beneficial, there is also a need to acknowledge studies that report otherwise, which can give us a fuller understanding of the mechanistic actions of the drug. In contrast to the results here, MitoQ has been reported to cause damage to mitochondria in mouse kidney tissue (Gottwald et al., 2018), and can instead increase ROS production in some cancer cells (Pokrzywinski et al., 2016). It is likely that these ill-effects could arise through improper dosage, and effects are also dependent on the inherent oxidative state in different conditions, which would be different for different diseases.

The antioxidant paradox

Beyond MitoQ, these paradoxical observations seem to be also related to a much broader issue prevalent in the field of antioxidant research, in that whilst antioxidants seem to show promise in controlled animal or *in vitro* oxidative stress-related models, these strategies have not had much success in the clinics. This has been termed as the “antioxidant paradox” (Halliwell, 2000).

Although ROS are involved in the pathophysiology of various diseases, they also act as important mediators of normal signalling processes. Thus, the indiscriminate lowering of the levels of oxidative stress could be damaging, tipping the delicate redox balance required for normal cellular functions. There needs to be a careful balance between ROS and antioxidants, especially so in the case of pregnancy, where a slight pro-oxidant state may be necessary (Fig 6.20). Thus, there is a need to determine the optimal dosage of antioxidants to use (Finkel and Holbrook, 2000, Poljsak et al., 2013). In the context of this study, dosage would also be dependent

on release patterns of MitoQ from the nanoparticles, and it is thus necessary to measure the concentration of MitoQ in the maternal brain, liver, plasma and foetal plasma. This can be achieved using LC-MS, where protocols have already been developed by various groups (Li et al., 2007).

At the same time, reversing oxidative stress is also clearly more complicated than simply the addition of an antioxidant. This is further compounded by the fact that each species of ROS (either a hydroxyl radical, superoxide, or nitric oxide radical) have different chemical properties and reaction rates (Halliwell, 2013). Likewise, antioxidants do not all work in the same manner, and administration of one type of antioxidant may result in compensatory actions in other endogenous antioxidant systems, which can be difficult to control for.

It has become increasingly clear that antioxidants may not have any effectiveness (or perhaps, can be counter-effective) unless there is a pre-existing deficiency or compromise (Fig 6.20). This may mean that the antioxidant intervention should only be administered to those at risk of disease, or those that already have a perturbation in the oxidative stress status. In this study, the treatment was given on GD16, the same day as when where the chronic stressor began. In reality, this may not be a very sensible method of therapeutic intervention, as it seemed impractical to be administering a treatment before or around the same time the chronic stressor is beginning. Predicting these “high risk groups” with oxidative stress perturbations will also be much harder in human populations, and it may therefore be challenging to translate the findings in this study to the clinic, precisely as postulated by the “antioxidant paradox”.

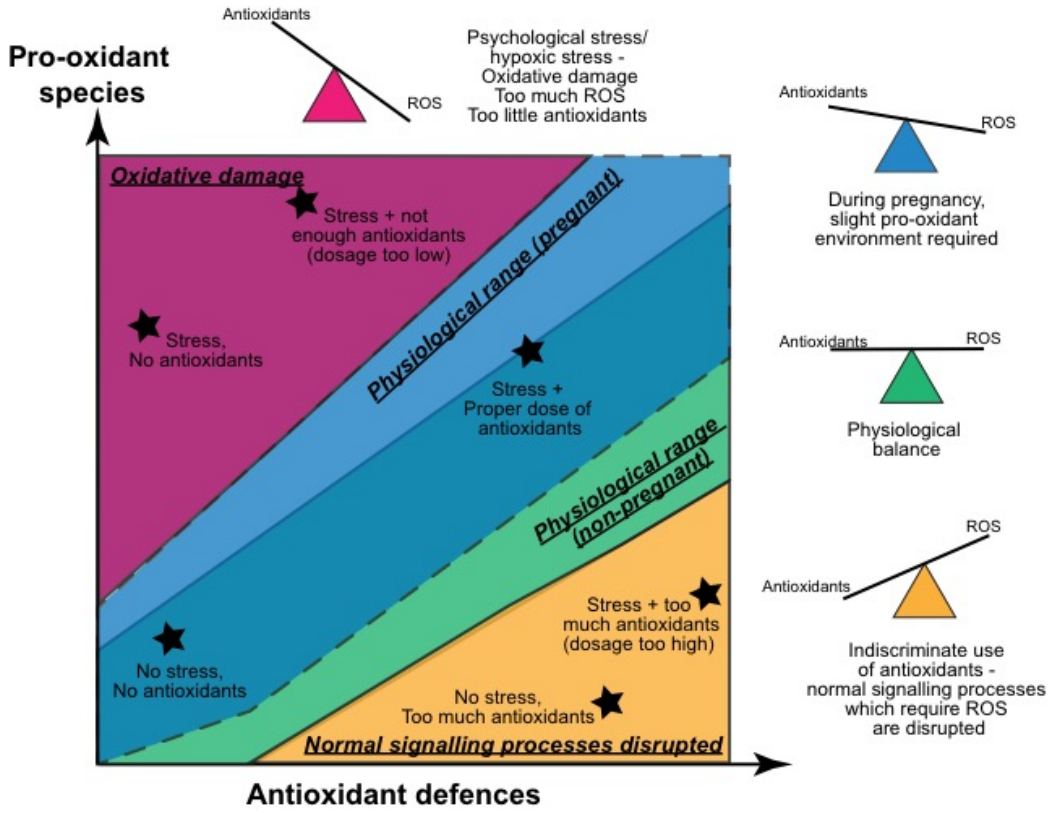


Fig 6.20: Importance of achieving the pro-oxidant vs antioxidant balance. Schematic showing how a balance between pro-oxidant and antioxidant species is needed to ensure optimal cellular functioning. When ROS exceeds antioxidant defences (pink zone), for instance, following psychological stress, oxidative damage to cellular components occurs. Pregnancy represents a constant state of mild oxidative stress (blue zone), and there is slightly greater levels of oxidative stress compared to the non-pregnant state (green zone). This pro-oxidant state is presumably required for adaptations to pregnancy. When antioxidants are given indiscriminately or in an inappropriate dose, it could also result in the disruption of normal cellular processes, which rely on ROS (yellow zone). Too much or too little antioxidants can therefore be harmful, and a careful titration of antioxidant dosage is required in order for interventions to be effective.

Possible side-effects of nanoparticles

Another major drawback in this study not yet mentioned, is the use of saline instead of NPs as vehicle, and the effects of NP injection alone were not investigated. Whilst the unique size and physical and chemical properties of nanoparticles confers it advantage in terms of accessibility to target organ/ tissues and penetrability into cells/ organelles, it also gives it the propensity to modify various biological processes and possibly cause harm (Nel et al., 2006). Depending on the type and formulation of NPs, they can cause toxicity, either from the constituents of the NPs itself, or from its degradation products (De Jong and Borm, 2008). Ironically, one of the harmful effects of NPs can be the increase in oxidative stress and cellular DNA damage (Nel et al., 2006). Also, despite being sequestered in layered "barrier" organs like the placenta or the gastrointestinal tract where they are unable to get past the first layer of cells, NPs can still induce downstream signalling processes which can potentially harm other surrounding cells without the need to be physically present in these cells (Bhabra et al., 2009).

However, it has been previously established that the formulation of NPs used in this study (γ-PGA-Phe) do not have toxic effects on cells, and upon injection in mice, do not provoke any injury to the tissue or activate any NP-specific immune response (Khalil et al., 2017). It was further observed in our studies that the rats did not present any discomfort or tissue damage at the site of injection.

Moving forward, there needs to be more thorough studies analysing the distribution and degradation patterns of NPs, before the drug can be brought into the clinic as a therapeutic invention. This could be carried out with the use of the fluorescent tagged NPs, for instance (Phillips et al., 2017). Maternal tissues could also be collected after weaning to study the long-term effects (if any) of NPs on the mother. If the efficacy and long-term safety of these NPs can be ascertained, these NPs can also potentially be used as a drug delivery system for other drugs (Refuerzo et al., 2017).

Despite these limitations and confounds in the experimental set up, this study adds on to the list of conditions for which maternal MitoQ-NP has been effective against, for instance, the effects of preeclampsia (Scott et al., 2018), the effects of gestational hypoxia on offspring neuronal characteristics (Phillips et al., 2017) and cardiac abnormalities (Aljunaidy et al., 2018), and most recently, DNA damage in a

mouse model of childhood leukemia initiation (Mansell et al., 2019). Additionally, maternal administration of MitoQ-NP to pregnant dams signifies the first time any maternal interventions have been attempted in this model of social stress, as previous forms of intervention (e.g. neurosteroid administration) were often targeted at the offspring after they were born and in adulthood (Brunton et al., 2015).

6.4.10 Conclusions and future work

The ability of MitoQ-NP to partially rescue the deleterious effects of maternal social stress in this study provided strong evidence that oxidative stress is involved in the transmission of prenatal stress from mother to foetus. Nonetheless, it has also been proposed that in spite of the generally positive results observed here, antioxidant therapy is not a magic bullet that can prevent all the negative effects of prenatal stress, as indiscriminate use can in fact be harmful.

Additionally, as proposed by Ghezzi et al (2017), given the centrality of ROS in various processes, it is actually not surprising to find that most disorders do indeed involve oxidative stress (Ghezzi et al., 2017). “Oxidative stress” can be contributed by a broad range of cellular processes and involves various mediators and players. Insofar as the conclusion “prenatal programming is mediated by oxidative stress” stands, a more thorough understanding of specific mechanisms that constitute “oxidative stress” is required for this conclusion to be of mechanistic and therapeutic significance. This is especially so for pregnancy-related disorders, as processes regulating oxidative stress following maternal stress are not intrinsically different from those observed in normal pregnancies, where a mild state of oxidative stress exists (Jauniaux et al., 2006). This study also offers an alternative way of viewing gestational stress, in that a stressed pregnancy represents an end of a continuum that is common to all pregnancies, and differs from normal pregnancies merely in terms of the magnitude of the oxidative stress processes. As such, any form of intervention targeting such processes need to be implemented with care.

A few suggestions on possible future work have been proposed throughout this discussion. To summarise, in order to strengthen the conclusions of this study, one can (i) repeat the experiment to investigate additional oxidative stress markers, (ii) quantify MitoQ, as ultimately, successful application of MitoQ-NP in the clinics will require greater understanding of the pharmacological properties of the nature of the drug, including their rates of absorption, tissue distribution and metabolism, (iii) run

large scale metabolomics studies to determine the identity of the placental secretions (and then in the subsequent stages, investigate the effects the depletion of these specific molecules, which can better prove mechanistic action).

Given the limitations of using antioxidants as an intervention, this discussion also identified a few other possible downstream targets that can be investigated, for instance, transcription factors like Nrf2, which may control the interaction between steroidogenesis and oxidative stress, or mediators of the serotonin signalling pathway, identified as being important in regulating stress coping behaviour. In fact, Nrf2 agonists are currently being used to treat multiple sclerosis and seem to be more successful in clinical trials when compared to antioxidant therapies (Dodson et al., 2019, Cuadrado et al., 2019). Additionally, in line with what is proposed in chapter 4, this study has also shown that alterations in GABA transmission is involved in the mediating the outcomes of prenatal stress, and interventions aimed targeting the GABA neurotransmission during development might also be of therapeutic value (reviewed in (Braat and Kooy, 2015)).

All in all, despite its focus on “oxidative stress”, this study revealed how multifactorial the possible contributors to foetal programming truly are, which is not surprising given that that “oxidative stress” and ROS signalling are involved in almost every single cellular process. Additionally, although this study initially set out to investigate mechanisms of prenatal stress beyond the HPA axis, towards the end of the chapter, it became clearer the HPA axis and neuroendocrine system cannot be completely disregarded, as any kind of intervention is likely to also affect aspects of the HPA axis. Eventually, an integrational and multi-level and -system investigation is needed, where data from human studies, *in vivo* animal models, and *in vitro* molecular and cellular studies from various systems are considered in parallel, in order to arrive at a better understanding of how prenatal social stress is being transmitted.

Chapter 7: General Discussion

The overarching aim of this thesis was to build on existing knowledge and further investigate the mechanisms underlying the outcomes of prenatal stress. To recap, this thesis had three broad aims. The first aim was to first develop a LC-MS method for the quantification of steroids, followed by addressing two questions to further elucidate mechanisms underlying the developmental programming of diseases in adulthood: “What are the mechanisms that are transmitting the stress signals from the mother to the foetus to result in foetal “programming”?” (Aim 2) and “what are the mechanisms underlying the expression of “programmed” phenotypes in the prenatally stressed adult offspring?” (Aim 3). Aim 2 allows for the formulation of interventions that can *prevent* such outcomes, while Aim 3 allows for the formulation of interventions that may possibly *reverse* such outcomes.

Following the successful development and validation of a LC-MS technique (Chapter 3; Aim 1), the concentrations of neuroactive steroids between control and prenatally stressed offspring were then compared, where it was determined that modest deficits in neuroactive steroid production exist in PNS offspring when compared to control offspring, following exposure to acute swim stress (Chapter 4, Aim 2). The same LC-MS method was then used to investigate the role of steroids, especially glucocorticoids, in the transmission of stress signals from mother to foetus (Chapter 5, Aim 3). Using a maternal targeted antioxidant, Chapter 6 then explored the role of oxidative stress and the placenta in the transmission of stress signals (Aim 2), and additionally, also investigated some of the neurochemical mechanisms that underlie adult offspring behaviour (Aim 3).

The discussion first provides a compilation of the key findings across all chapters. Some commonalities (e.g. the presence of sex differences) will be further examined. Overall limitations and the translatability of the work are then considered, and lastly, future directions that can be taken to better understand the mechanism mediating the outcomes of prenatal stress will be explored.

7.1 Key findings of the thesis

7.1.1 Foetal "programming": What are the mechanisms involved in transmitting the stress signals from mother to foetus?

The mechanisms mediating the transmission of the stress signals were examined in Chapter 5 and 6 (Figure 7.1). The widespread assumption in the field of foetal programming is that stress signals are communicated to the foetus by the direct diffusion of maternal glucocorticoids through the placenta, resulting in foetal overexposure to maternal glucocorticoids (Wyrwoll and Holmes, 2012, Cottrell and Seckl, 2009). However, in this thesis, it was shown that whilst there was an activation of the maternal HPA axis, the was no evidence for increased corticosterone concentrations in the foetal brain. Furthermore, there was also an increase in 11β-HSD2 mRNA expression in the placenta, possibly indicating increased protection from the "glucocorticoid barrier". The role of maternal glucocorticoids in foetal programming therefore does not involve a direct crossover, and is likely to occur through complex mechanisms involving glucocorticoid modulation of other aspects of maternal physiology. There were also no major changes in the concentrations in a panel of neuroactive steroids in the foetal brain following gestational stress. Alteration of steroid production machinery in the foetal brain during development therefore does not seem to be involved in adverse foetal programming.

Apart from an increase in maternal glucocorticoids following gestational stress, there was also an increase in oxidative stress in the maternal compartment and in the placenta following gestational stress. It was suggested that the stressed placenta can secrete damaging factors into the foetal circulation, and through an *in vitro* experiment, it was revealed that these placental secretions from stressed placenta have the propensity to alter neuronal characteristics. Most importantly, the use of a maternal antioxidant prevented placental secretions and foetal plasma from altering these neuronal characteristics. These, as yet unidentified placental secretions that are dependent on maternal/placental oxidative stress are therefore a plausible mechanism that communicate stress signals from the mother to the foetus, and the next step is to determine the exact identity of these damaging factors using untargeted metabolomic/proteomic methods.

Putting the results from Chapter 5 and 6 together, a speculative hypothesis of how prenatal stress may be transmitted to the offspring is also presented in Figure 7.2. These two mechanisms are not mutually exclusive, and could each represent part of the puzzle regarding the mechanisms mediating the outcomes of prenatal stress. Corticosterone may exert its effects through the activation of a downstream cascade that increases cellular oxidative stress, rather than through direct crossover of the placenta. Nonetheless, this remains only speculative and will need to be tested with further experiments involving maternal adrenalectomy with substitutive corticosterone therapy for instance.

As for the prevention of these outcomes, this work has shown that maternal antioxidant therapy with a nanoparticle drug delivery system was effective in rescuing many of the deficits associated with PNS in the rat model (Chapter 6). Mechanisms regulating the transmission of stress are likely to be complex, and on a translational viewpoint, pharmacological intervention may not be always feasible in human populations. Nonetheless, increased understanding of the roles corticosterone and oxidative stress may play in the transmission of stress signals can guide intervention approaches, which need not be pharmacological in nature, and may stem from environmental or dietary, or in the case of humans, psychological or societal perspectives.

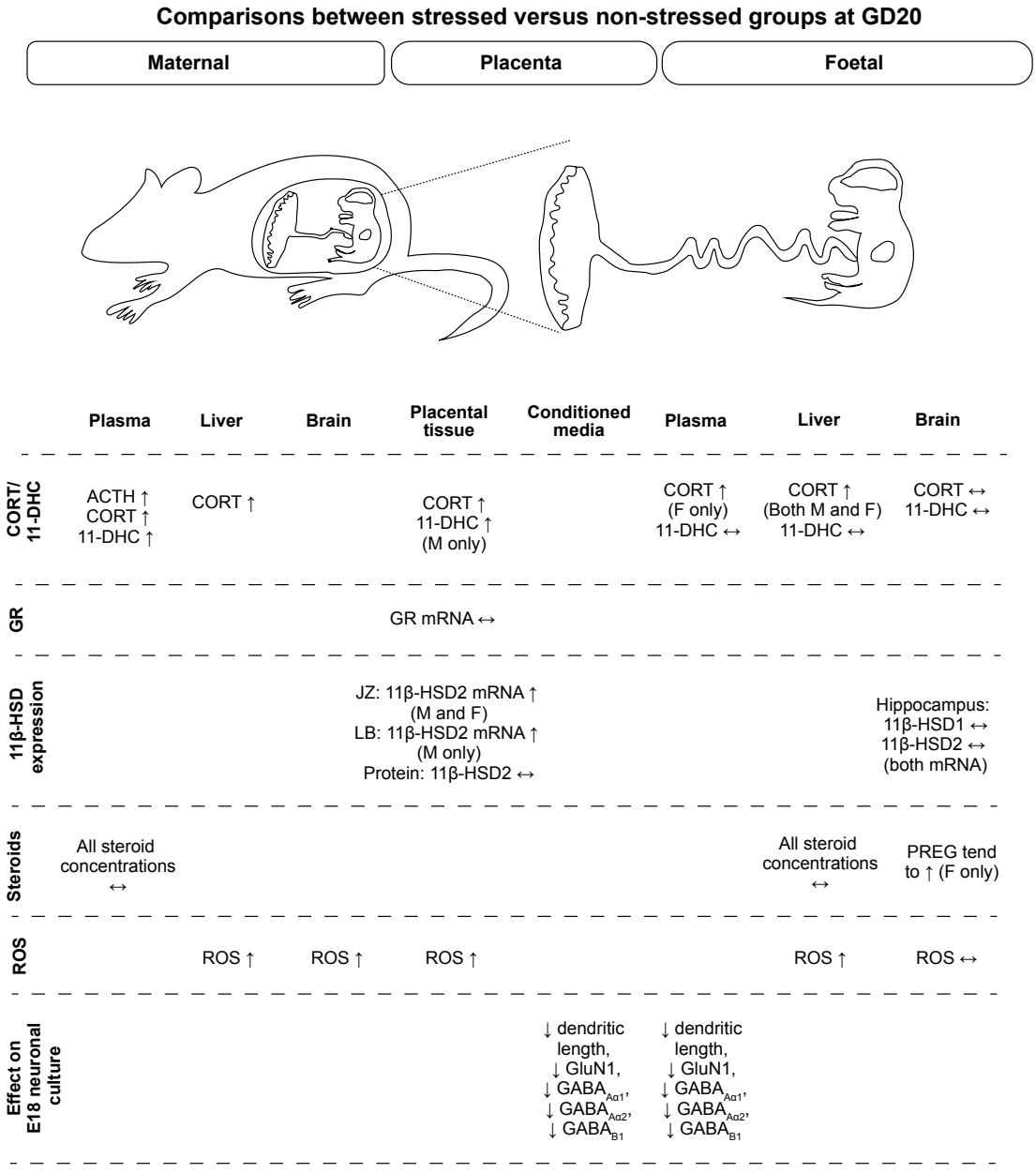


Figure 7.1: Summary of differences in stressed groups as compared to non-stressed controls at GD20 following social stress. Data summarised from Chapters 5 and 6. An up arrow (↑) denotes that stressed group exhibited an increase in the parameter as compared to controls, while a down arrow (↓) denotes that stressed group exhibited a decrease in the parameter as compared to the control group. A double-ended horizontal arrow (↔) indicates no differences between the stressed and non-stressed groups.

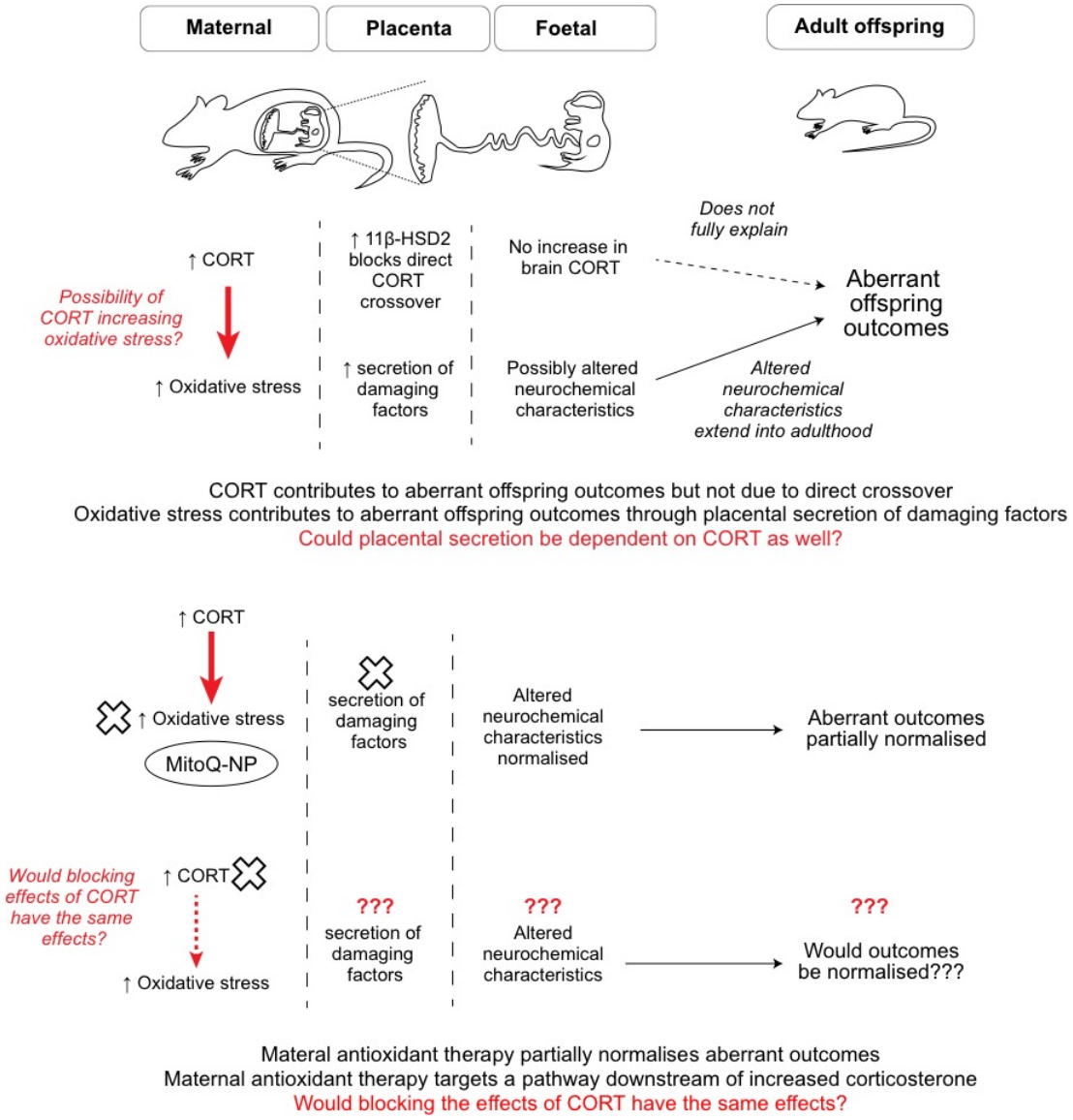


Figure 7.2: Conclusions and proposed mechanisms mediating the transmission of stress signals from mother to foetus. CORT: corticosterone

7.1.2 What are the mechanisms underlying the expression of "programmed" phenotypes in the adult prenatally stressed offspring?

The second focus of the thesis investigated the mechanisms that underlie the observed phenotypical outcomes in the PNS adult offspring (e.g. heightened anxiety and HPA axis dysregulation), and also characterised additional behavioural outcomes related to depressive-like behaviour (Figure 7.3). Due to the involvement of GABA neurotransmission in many mood and affect related disorders, it was first hypothesised in Chapter 4 that deficiencies in the production of $GABA_A$-modulating neuroactive steroids could underlie the anxiety and HPA axis dysregulation observed in PNS offspring. However, no differences in the concentration of these neuroactive steroids between control and PNS rats were observed under basal levels. Nonetheless, following acute swim stress, PNS offspring seemed to exhibit lower THDOC, but not allopregnanolone production, in various brain regions, indicating that there could be a deficit in metabolism pertaining to the DOC pathway, rather than the progestogen pathway in PNS offspring. Although the concentrations of these $GABA_A$ allosteric modulators were largely not affected in the PNS groups, it was shown in Chapter 6 that the expression patterns of the $GABA_A$ receptors were altered, suggesting altered baseline GABA neurotransmission could still be a contributory factor underlying the behavioural outcomes in the PNS offspring. Changes in $GABA_A$ subunit expression were also sex- and region dependent, while absolute neuroactive steroid concentrations were generally found to be greater in the female brain as compared to the male brain.

Although attempts to reverse these outcomes during adulthood were not carried out in this study, decreased $GABA_A$ receptor expression may explain why neuroactive steroid supplementation in adulthood is effective in normalising HPA axis responses to stress (Brunton et al., 2015). As PND30 brains were studied for neurochemical and neuroanatomical changes, this also indicates that many of these neurochemical changes are present before puberty, and that the trajectory for behaviour deficits seem to be already determined before adulthood. In order to further investigate whether these changes begin at an earlier stage, neurochemical characteristics in foetal brains could be investigated, and this would then possibly allow for the alteration of the trajectory of development before negative behavioural symptoms arise.

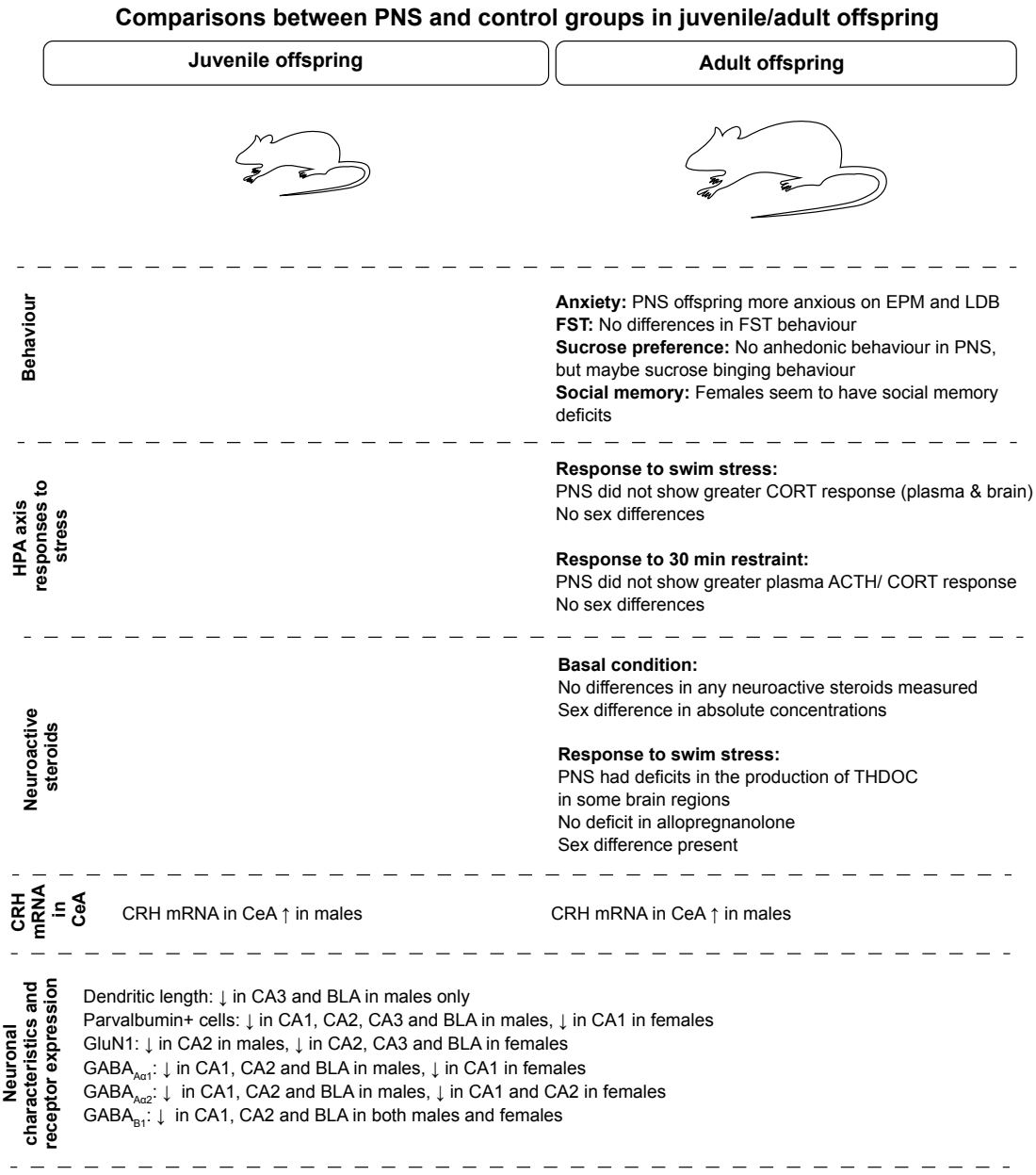


Figure 7.3: Summary of differences in stressed PND30 and adult offspring compared to respective non-stressed controls. Data summarised from Chapters 4 and 6. (↑) denotes that stressed group exhibited an increase in the parameter as compared to controls, while (↓) denotes that stressed group exhibited a decrease in the parameter as compared to the control group.

7.2 Sex differences as a recurring theme

In this study, sex differences in the brain and behaviour was a recurring theme that was observed across all chapters, for most of the parameters tested. Sex differences occurred on two levels, basally (i.e. males and females are biologically different), and on a level altered by changes such as stress or the prenatal environment (i.e. males and females respond differently to environmental stimuli).

Baseline sex differences fit into the organisational-activational framework of sexual differentiation, which posits that sex hormones permanently organise the CNS during early development; while in adulthood, sex hormones serve to activate these systems that have been organised prenatally (Arnold, 2009). At GD20, testosterone concentrations were very different in males and females. This is a critical stage of sexual differentiation, where the developing foetal brain is in the process of being altered by the organisational effects of testosterone and oestradiol, after testosterone conversion via aromatase. Regretfully, oestradiol could not be detected using this LC-MS method, but concentrations of other neuroactive steroids (e.g. progesterone and DOC and their metabolites) could be quantified. It was found that although concentrations of these neuroactive steroids were not different in the brains of male and female foetuses (Chapter 5), a sex difference became apparent in the adult brains, where females generally show greater concentrations (Chapter 4). The difference in baseline neuroactive steroid concentrations in adult rat brains can be considered a consequence of both “organisational” (in that it is a permanent and long-lasting effect arising from foetal development) and the constant “activational” effects of sex hormones (in that it is dependent on the continued production of sex hormones from the gonads). According to the organisational-activational framework, there would also exist many other important sex differences in the brain (e.g. anatomical or circuitry differences), all of which may possibly contribute to differential responses between males and females to challenges, either in adulthood or during development (Bangasser and Wicks, 2017). Additionally, apart from this basic framework, sex differences in placental function also exist due to the action of sex chromosomes, which can also contribute to the baseline sex differences observed in adulthood (Bale, 2016).

In this work, there were indeed differences in how males and females responded to acute stress (Chapter 4), which partly recapitulated the results reported in a

previously published study (Sze et al., 2018). Prenatal stress also resulted in different outcomes in male and female offspring (Chapter 5 and 6), which corroborates general observations in various developmental programming models (Aiken and Ozanne, 2013). These sex differences are a result of complex interactions between the "organised" sex differences in the brain of the adults, including neuroactive steroid concentrations, as well as other factors like the inherent differences between XX and XY placentae (Bale, 2016). Although it seemed like males presented higher vulnerability to developmental compromises in this study, which was similar to that proposed in some studies (Gobinath et al., 2014), comparisons between male and female vulnerability eventually depends on the nature of the stressor and on the offspring trait that is being investigated (Glover and Hill, 2012, Weinstock, 2007).

Ultimately, regardless of which sex seem more "vulnerable" to the effects of stress, it is critical to include both males and females in the dataset and to analyse results by sex, as has been done in most parts of this thesis. This has two important implications. Firstly, it can further shed light on the mechanisms of observed phenomena, and secondly, it can also have therapeutic implications (McCarthy et al., 2012). Since stress processes heavily involve steroid hormones, by completely omitting one sex, fundamental misinterpretation of the experimental results may occur. Even if the phenotypical endpoints do not show a sex difference, the underlying physiology of males and females are still by and large different, and this sex convergence is likely to reveal the presence of compensatory mechanisms (De Vries, 2004, McCarthy et al., 2012). By not taking sex into consideration, there can be inaccuracies in mechanistic conclusions, which then could result in dire consequences in terms of formulating interventions, especially for the population that is understudied (in most cases, females).

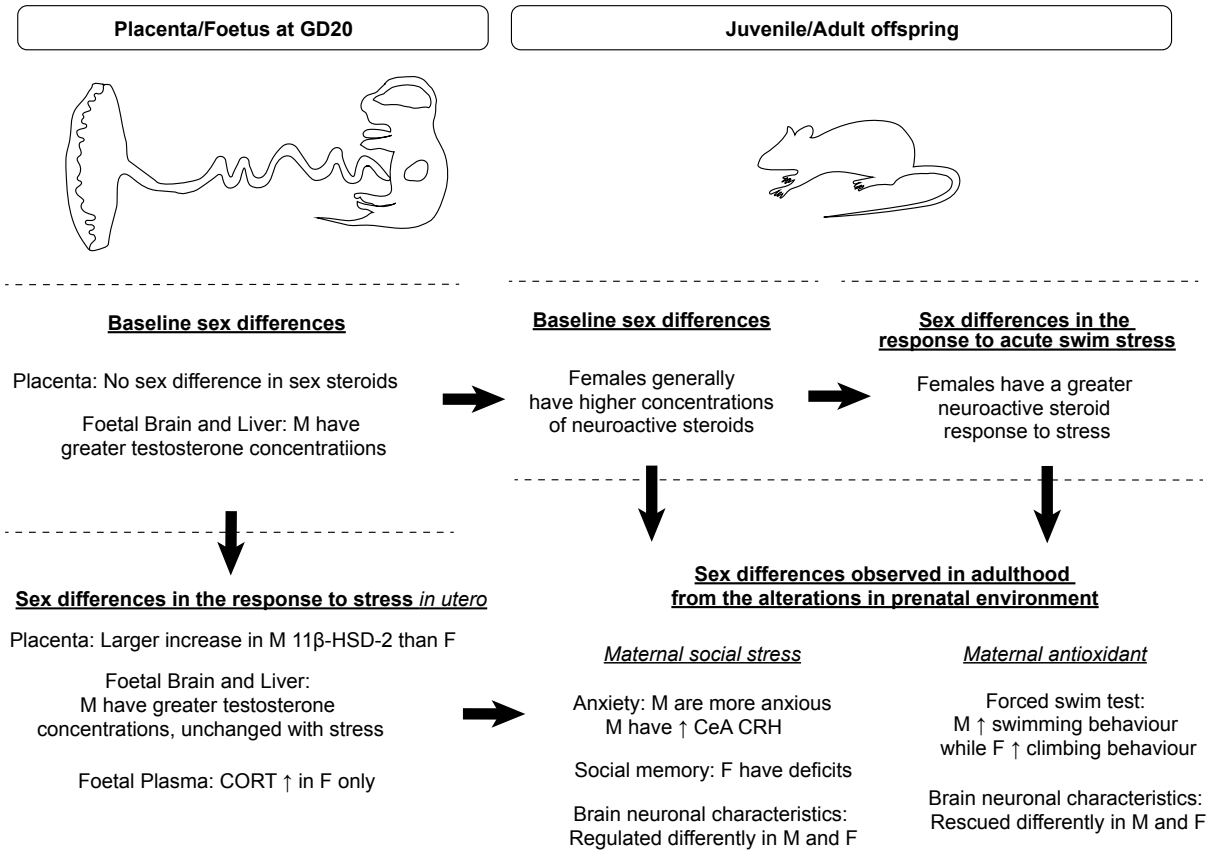


Figure 7.4: Sex differences present in this study. Sex differences in the response to acute stress, or to stressors in early development are dependent on the inherent sex differences at baseline between males and females. It is of note that other than the parameters observed here, anatomical, neurochemical and circuitry differences exist in males and females that are all a consequence of the organisation-activational actions of sex hormones, and likely underlie observed behavioural/neuroanatomical outcomes following stressors.

7.3 Limitations of this work

The limitations of each experiment, whether methodological or interpretational in nature, have been discussed separately in each chapter. Three general themes arise: (i) experimental techniques, (ii) experimental design, and (iii) reproducibility of the experiments.

7.3.1 Experimental techniques

This thesis consisted of a wide array of experimental techniques, from traditional methods used in physiology studies (e.g. *in situ* hybridisation and radioimmunoassays) to new techniques such as LC-MS and nanotechnology drug delivery systems in an attempt to answer different aspects of the same biological question. Whilst *in situ* hybridisation is a highly sensitive method used to detect mRNA expression in an anatomically specific manner, a change in mRNA expression does not equate to changes in protein expression, therefore immunohistochemistry could also have been additionally carried out to verify altered mRNA expression results translates to differences in protein expression. The HPA axis has also been investigated only by the quantification of corticosterone, and functional assessments (e.g. using the dexamethasone suppression test) or the expression of the receptors (e.g. GR) could be carried out to determine if there were any deficits in negative feedback mechanisms. Apart from investigating these biological markers in the brain, this work also correlated these neurochemical/endocrine observations to functional consequences using behavioural tests, and the limitations of each test have been discussed in Chapter 6.

7.3.2 Experimental design: Descriptive versus mechanistic science

The second drawback is that some experiments in this thesis were purely "descriptive" but not "mechanistic" in nature (Casadevall and Fang, 2009). "Mechanistic" studies require the determination of causal relationship between event A and B, and in general, three kinds of experiments need to be performed. Firstly, A and B should be measured without any manipulations, to first show a correlation under biologically realistic conditions. Negative manipulations (e.g. gene knockouts or receptor antagonists) can then be performed, where the magnitude/intensity of A is decreased, and the effects on B measured. However, negative manipulations typically cannot distinguish between background factors necessary for the effect and a genuinely triggering cause (Bickle, 2015), thus a third kind of experiment, where

positive manipulations are used (e.g. overexpressing or receptor agonists of A) should also be carried out. Only when all three kinds of experiments consistently point towards the same conclusion, can a causal mechanism confidently be determined (Silva et al., 2013).

The studies in Chapter 4 and 5 were descriptive in nature, as concentrations of neuroactive steroids were measured under biologically realistic conditions and no manipulations were carried out. Given the pervasive nature of neuroactive steroids and the fact that they are made in multiple regions of the brain by the same enzymatic mechanisms, certain negative manipulation methods (e.g. ablating the tissue that produces the steroid or giving a receptor antagonist) would be difficult to apply, or may not result in any conclusive data. More sophisticated methods with regional specific conditional gene knockout/ adenoviral delivery of genes to manipulate steroid synthesis may be required to tease apart the exact role of specific neurosteroids in regulating specific functional outcomes. Nonetheless, this "descriptive" study is still important in that it was necessarily, as a first step, to establish if there were any differences on a baseline level before any manipulations were given.

In Chapter 6, negative pharmacological manipulation with the use of an antioxidant indicated that ROS production (the causal agent) was necessary and sufficient to cause negative programming effects. However, it also suggested this that experimental method can have limitations as it may manipulate a system beyond the limits of normal functioning (in this case, normal ROS signalling could have been affected). Therefore, results should still be analysed with caution.

7.3.3 Reproducibility

The third limitation is that there exists certain instances where the results in this study were not reproducible from previous published studies, especially in the case of measuring HPA axis responses to stress. Possible explanations of these discrepancies have been separately discussed in each chapter. In general, the neuroendocrine response to stress and behavioural assays are extremely sensitive to environmental changes, and even changes to bedding, diet, or drinking water in different animal units can result in large impacts on experimental results due to the alteration of the gut microflora (Franklin and Ericsson, 2017).

Additionally, there were several instances in this work where trends towards statistical significance were observed (p-value between 0.05 and 0.10). A repeat of the same experiment, with increased n numbers to increase power, are required to verify these results, but these are not always feasible due to cost or logistical issues. However, it is still of interest to report and discuss these data in this work albeit with accompanying nuances and caveats, as it shows the dynamic nature of stress responses. Throughout the thesis, different offspring from each dam were taken for each data point, which reduces 'within' litter effects, whilst individual data points in each group have been plotted. Together, they reflect the heterogeneity of offspring outcomes, but also the variability in the stress responses of the pregnant dam. The inherent variability of stress responses are known and not new, as there are often puzzling reports on how variable results can emerge even from virtually identical experimental designs (Murthy and Gould, 2018), and can be attributed to subtle differences related to baseline housing and testing differences, natural variation in responses to stress and maternal care, and also individual variabilities that are present. Nonetheless, these are taken into account by presenting each of the individual data points for each group where possible.

7.4 Complexity of the mechanisms mediating the outcomes of prenatal stress

It is important to acknowledge again that stress responses, whether acute or chronic, are modulated by a wide range of physiological systems and are inherently complicated. In as much as simplicity in experimental design is required to properly tease apart mechanisms, complexity exists in neuroendocrine mechanisms, and these supposed "caveats" could actually be key mechanistic factors that can explain a phenomenon (Karatsoreos, 2016).

The concept of allostasis, introduced in section 1.2.2, may provide a holistic framework for better understanding the mechanisms mediating the outcomes of prenatal stress. To recap, allostasis refers to the adaptive changes that occur following stress. Allostasis therefore can be occurring both in the pregnant dam in response to five days of chronic stress, and also in the PNS offspring, where stress experienced *in utero* constitutes part of the allostatic load. For the pregnant rat, pregnancy itself can already be considered an allostatic state due to alterations in its physiological systems, not only in HPA axis functioning but also metabolism,

immune and vascular function (section 1.4.1). Thus, when an additional allostatic load (here, chronic social stress) is superimposed on this "allostatic state", it could further push the allostatic load past a tipping point to result in "allostatic overload" (Russell and Brunton, 2019). For instance, the placenta, which exists in a mildly oxidative state (Myatt and Cui, 2004), was subject to further allostatic load (in the form of further oxidative stress) induced by chronic social stress, and "allostatic overload" may manifest as the secretion of damaging factors into the foetal circulation (Chapter 6). In the PNS offspring, this chronic stress *in utero*, which exposes the foetus to damaging placental factors, could represent part of the "wear-and-tear" that it will experience throughout its life course (McEwen, 2004). This allostatic load that occurs early in life could set the trajectory towards poorer adult outcomes and the development of mood-related disorders during adulthood. In both cases, chronic gestational social stress pushes the allostatic state closer to that of an allostatic overload, thereby increasing the risk of pathology.

The allostatic load is contributed by multiple players spanning different levels of organisation, and could include structural remodelling of neural architecture, changes in baseline levels of neurotransmitters and receptors, or their intra-cellular and extracellular modulators, resulting in altered HPA axis function, among many others (McEwen et al., 2015). Beyond the main mediators of the HPA axis (CRH, ACTH, glucocorticoids), there also exists a diverse array of neuroactive steroids, which may each play a different role in the regulation of stress responses (reviewed in Chapter 4). This diversity of steroid mediators was addressed in Chapter 3, with the development of a technique to quantify a panel of steroids that are in involved in mediating stress responses. Although steroids and the HPA axis are the focus of the initial part of the thesis, the change of one allostatic system could lead to knock-on changes in other mediator systems (inflammatory molecules, cytokines, reactive oxygen species), thereby affecting the final outcome. Chapter 6 then addressed some of these other mediator systems, such as oxidative stress and reactive oxygen species. Maternal antioxidant treatment in this case, can also be viewed as an intervention that can alleviate the allostatic load, bringing it further away from the tipping point. This also allows us to integrate the findings from previous studies, into a single model in order to further our understanding of the mechanisms mediating prenatal programming and offspring outcomes. For instance, although PNS female offspring may not have deficits in allopregnanolone concentrations *per se*, the use of allopregnanolone treatment in adult female PNS offspring in the 2015 study

(Brunton et al., 2015) could be viewed as a way in which allostatic load can be reduced, thereby rescuing aberrant outcomes.

7.5 Translatability of the research

Given the complexity of the mechanisms mediating prenatal stress (section 7.4) and the various caveats in the study (section 7.3), this inevitably raises the question - how translatable is this work to humans?

Firstly, one could argue that the physiology of the rat and humans are inherently different, and that maternal glucocorticoids may not be regulated in the same way. As mentioned in the introduction, there are several differences between the rat and human placenta, for instance, the lack of placental CRH production in rats (Alcántara-Alonso et al., 2017). In human epidemiology studies, it has also been recognised that HPA axis outputs (i.e. cortisol) were not robust biological measures of maternal distress, casting doubt on the significance of glucocorticoid metabolism in the transmission of stress signals in humans (Golden et al., 2011). However, the purpose of using animal models is not to completely transfer the results to the human condition. As long as the model generates logical data, it is meant to complement studies in humans in order to move the field forward (Bonney, 2013).

Inherent to all *in vivo* animals models is also the conundrum that whilst the careful control of variables is a necessity in deriving mechanistic knowledge, this degree of control is precisely the obstacle to the translatability from bench to bedside, since humans do not exist in controlled environments (Dipietro, 2012). This is especially pertinent for the field of stress research, as socioeconomic factors like poverty are in fact the main contributors to poor offspring outcomes (Blair and Raver, 2016). However, understanding basic physiological mechanisms could be the starting point in guiding interventions (in a medical aspect) or other changes (e.g. social or political) which can positively impact those at risk.

Again, the allostatic framework may allow for the better translation of *in vivo* studies, as it allows for the socio-psychological and physiological phenomena to be linked together (Sterling and Eyer, 1988). An allostatic load index which includes various biomarkers can be formulated (Juster et al., 2010), and this has been done for pregnant mothers for the risk of preterm birth and preeclampsia (Barrett et al., 2018) as well as for preterm infants (Casavant et al., 2019). Placental markers (Hodyl et

al., 2017) and other factors such as socio-economic factors (Seeman et al., 2010), or ethnic and sex-based differences (Rodriquez et al., 2019) can also be taken into consideration. Most recently, a rat cumulative allostatic load measure (rCALM) has been proposed as a preclinical translation tool to more relevantly assess the burden of chronic stress in the rodent model (McCreary et al., 2019). Eventually, processes related to stress and pregnancy should be studied using a multi-level approach comprising of different expertise from different fields (i.e. not only in the biological but also psychological and socio-economic domains), in order to better understand how early life environment can shape birth outcomes (Dunkel Schetter, 2011).

7.6 Concluding remarks

All in all, this thesis analysed the role of glucocorticoids, neuroactive steroids, and oxidative stress in mediating the outcomes of chronic gestational social stress, using a rodent model. It highlights the importance of sex differences and steroid influences in the study of stress-related mechanisms, and proposes for the conclusions here to be viewed using the allostasis framework. It also demonstrates the nature of collaborative research, where expertise in chemistry and nanotechnology could be used to complement traditional physiological techniques. The thesis resonates with a commentary by Carl Woese in the article “A new biology for a new century”,

> *“Science is an endless search for truth. Any representation of reality we develop can be only partial. There is no finality, sometimes no single best representation. There is only deeper understanding, more revealing and enveloping representations. Scientific advance, then, is a succession of newer representations superseding older ones, either because an older one has run its course and is no longer a reliable guide for a field or because the newer one is more powerful, encompassing, and productive than its predecessor(s). Science is impelled by two main factors, technological advance and a guiding vision. A properly balanced relationship between the two is key to the successful development of a science: without the proper technological advances the road ahead is blocked. Without a guiding vision there is no road ahead; the science becomes an engineering discipline, concerned with temporal practical problems.” (Woese, 2004)*

Indeed, the field has progressed immensely since the first epidemiological associations carried out in 1989, which was first met with some scepticism. Exactly

30 years down the road, the field has expanded from reporting epidemiological associations to the dissection of molecular mechanisms, and is still continuing to progress at a tremendous pace (Limesand et al., 2019). This work represents only a tiny aspect of all the possible mechanisms underlying the foetal programming of adulthood diseases. Molecular mechanisms like epigenetics for instance, is a key player in foetal programming (Vo and Hardy, 2012, O'Donnell and Meaney, 2017), while the role of the gut microbiota and transgenerational transmission of prenatal stress may also provide interesting perspectives. To disentangle the contribution of each and every mediator system requires the individual pharmacological manipulation of each system, which is beyond the capability of any single laboratory. Similar to what has been done in this work, progress requires a concerted effort from various groups and researchers around the world, sharing technologies and insights to contribute to this pool of knowledge that has been built up over the past 30 years. The deeper and more integrated understanding of these mechanisms can then hopefully bring about new ways for the prevention, reversal, or management of negative birth outcomes, thereby bettering the lives of affected human populations.

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Appendix A: Two or three-way ANOVA on R-studio

TWO WAY ANOVA

Using the example of male plasma corticosterone in Chapter 3 (Figure 3.5A), where Factor 1 is Prenatal stress and Factor 2 is Acute Stress, generate Excel file with the following parameters and column names:

Filename: maleplasma; Column 1: Prenatal; Column 2: Acute; Column 3: Cort

Step 1: Visualising the data in order to determine if there is equal variance

```
boxplot(Cort~ Prenatal*Acute, data=maleplasma)
stripchart(Cort~ Prenatal*Acute, data=maleplasma, vertical=TRUE,
method="jitter", add=TRUE)
```

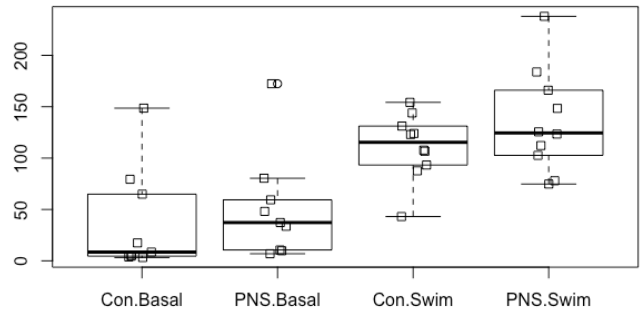

Figure A1: Example of a check for equal variance. The spread of the data and the presence of any outliers were noted.

Step 2: Fit into linear model and determine if there are problems with normality

```
options(contrasts=c("contr.sum","contr.poly"))
maleplasmacort <- lm(Cort~ Prenatal*Acute, data=maleplasma)
plot(maleplasmacort)
```

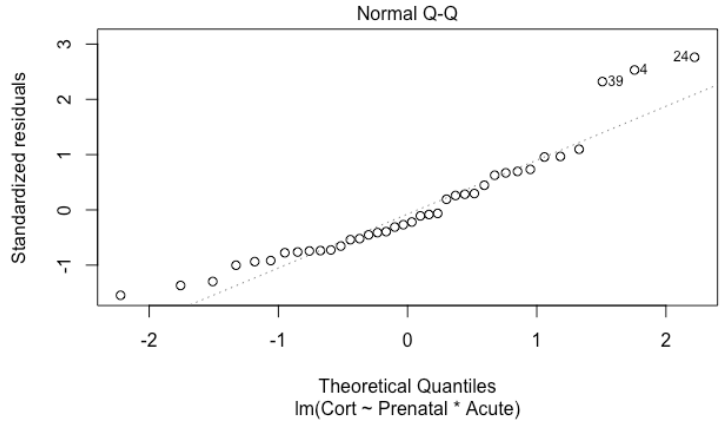

Figure A2: Example of a normality check. All data points should closely follow the straight line and should not looks curvilinear or S-shaped.

Step 3: Run two-way ANOVA

Install and load “car” package

```
Anova(maleplasmacort, type=”III”)
```

This will generate a read-out:

```
Anova Table (Type III tests)
Response: Cort
              Sum Sq Df  F value    Pr(>F)
(Intercept)   265771  1 122.1706 8.452e-13 ***
Prenatal        3317  1   1.5249    0.2253
Acute          59510  1  27.3559 8.631e-06 ***
Prenatal:Acute   246  1   0.1131    0.7387
Residuals      73964 34
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

A main effect of “Acute stress” is observed. There is no main effect of “Prenatal” stress nor Prenatal:Acute stress interaction.

Step 4: Run post-hocmultiple pairwise comparisons

```
with(maleplasma,pairwise.t.test(Cort,interaction(Prenatal,Acute),
data=maleplasma,p.adj="none",paired=F))
```

This will generate a read-out:

```
Pairwise comparisons using t tests with pooled SD
data:  Cort and interaction(Prenatal, Acute)

          Con.Basal PNS.Basal Con.Swim
PNS.Basal 0.53984   -         -
Con.Swim  0.00147   0.00785   -
PNS.Swim  6.1e-05   0.00039   0.26168

P value adjustment method: none
```

THREE WAY ANOVA

For three way ANOVA, similar techniques apply, but with three factors instead of two factors. Using the example of male and female plasma corticosterone, where Factor 1: Prenatal Stress, Factor 2: Acute Stress, Factor 3: Sex (i.e. data from Figure 3A and 3G), generate Excel file with the following parameters:

Filename: plasma;
Column 1: Prenatal; Column 2: Acute; Column 3: Sex; Column 4: Cort

Step 1: Visualising the data in order to determine if there is equal variance

```
boxplot(Cort~ Prenatal*Acute*Sex, data=plasma)
stripchart(Cort~ Prenatal*Acute*Sex, data=plasma, vertical=TRUE,
method="jitter", add=TRUE)
```

Step 2: Fit into linear model and determine if there are problems with normality

```
options(contrasts=c("contr.sum","contr.poly"))
plasmacort <- lm(Cort~ Prenatal*Acute*Sex, data=maleplasma)
```

Step 3: Run three-way ANOVA

Install and load “car” package

```
Anova(plasmacort, type=”III”)
```

This will generate a read-out:

```
Anova Table (Type III tests)
Response: Cort
                  Sum Sq Df  F value    Pr(>F)
(Intercept)       719630  1 179.0282 < 2.2e-16 ***
Prenatal              19  1   0.0047   0.94538
Acute             198517  1  49.3867 1.113e-09 ***
Sex                11980  1   2.9804   0.08869 .
Prenatal:Acute      3569  1   0.8879   0.34929
Prenatal:Sex        6113  1   1.5208   0.22162
Acute:Sex           9202  1   2.2893   0.13477
Prenatal:Acute:Sex  6760  1   1.6818   0.19895
Residuals         281375 70
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

A main effect of “Acute stress” is observed. There were no three way interactions observed. There were no interactions of neither Prenatal or Acute stress with Sex.

If variance is not equal and log transformation is required:

```
plasma$Cortlog <- log(plasma$Cort)
```

which generates a new column of log-transformed values.

Then proceed to generate linear model and run three-way ANOVA with log-transformed values.

```
options(contrasts=c("contr.sum","contr.poly"))
plasmacortlog <- lm(Cortlog~ Prenatal*Acute*Sex, data=plasma)
Anova(plasmacortlog, type=”III”)
```

Appendix B: Published paper “Sex-dependent changes in neuroactive steroid concentrations in the rat brain following acute swim stress” (Chapter 3)

Main text: Page -451- to -469

Supplementary information: Page -471- to -478-

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ORIGINAL ARTICLE

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Sex-dependent changes in neuroactive steroid concentrations in the rat brain following acute swim stress

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Sex differences in hypothalamic-pituitary-adrenal (HPA) axis activity are well established in rodents. In addition to glucocorticoids, stress also stimulates the secretion of progesterone and deoxycorticosterone (DOC) from the adrenal gland. Neuroactive steroid metabolites of these precursors can modulate HPA axis function; however, it is not known whether levels of these steroids differ between male and females following stress. In the present study, we aimed to establish whether neuroactive steroid concentrations in the brain display sex- and/or region-specific differences under basal conditions and following exposure to acute stress. Brains were collected from male and female rats killed under nonstress conditions or following exposure to forced swimming. Liquid chromatography-mass spectrometry was used to quantify eight steroids: corticosterone, DOC, dihydrodeoxycorticosterone (DHDOC), pregnenolone, progesterone, dihydroprogesterone (DHP), allopregnanolone and testosterone in plasma, and in five brain regions (frontal cortex, hypothalamus, hippocampus, amygdala and brainstem). Corticosterone, DOC and progesterone concentrations were significantly greater in the plasma and brain of both sexes following stress; however, the responses in plasma were greater in females compared to males. This sex difference was also observed in the majority of brain regions for DOC and progesterone but not for corticosterone. Despite observing no stress-induced changes in circulating concentrations of pregnenolone, DHDOC or DHP, concentrations were significantly greater in the brain and this effect was more pronounced in females than males. Basal plasma and brain concentrations of allopregnanolone were significantly higher in females; moreover, stress had a greater impact on central allopregnanolone concentrations in females. Stress had no effect on circulating or brain concentrations of testosterone in males. These data indicate the existence of sex and regional differences in the generation of neuroactive steroids in the brain following acute stress, especially for the 5α-reduced steroids, and further suggest a sex-specific expression of steroidogenic enzymes in the brain. Thus, differential neurosteroidogenesis may contribute to sex differences in HPA axis responses to stress.

KEYWORDS
5α-reductase, glucocorticoids, hypothalamic-pituitary-adrenal (HPA) axis, neurosteroids, progestogens, sex differences

1 | INTRODUCTION

Steroid hormones play a crucial role in regulating physiological responses to stress. They can exert their actions in target tissues by binding to classical steroid receptors, which then act as transcription factors in the nucleus to alter gene expression. Additionally, steroids can act in a nonclassical manner. Neuroactive steroids are active metabolites of classical steroid hormones that, independent of their source (ie, peripheral or central), exert rapid nongenomic effects on neuronal excitability by binding to membrane bound ion channel-linked receptors, thus influencing neurotransmission.[1] The brain itself can produce neuroactive steroids (referred to as "neurosteroids"), via local de novo synthesis from cholesterol or through the conversion of peripherally derived adrenal or gonadal steroids.[1,2] In support of this, the brain possesses many steroidogenic enzymes, including 5α-reductase and 3α-hydroxysteroid dehydrogenase (3αHSD) which are not only mainly expressed by glial cells,[3-5] but also found in neurones.[6] Furthermore, neurosteroids continue to be detected in the brain after combined adrenalectomy and gonadectomy,[7] providing evidence for local neurosteroidogenesis. Nevertheless, the synthesis of neurosteroids is reliant upon the expression of the prerequisite steroidogenic enzymes, which exhibit significant regional differences in the brain.[8]

The major neuroendocrine response to stress is mediated via activation of the hypothalamic-pituitary-adrenal (HPA) axis, which results in a net increase in circulating glucocorticoids following activation of corticotrophin-releasing hormone (CRH) neurones in the medial parvocellular paraventricular nucleus (mpPVN) and adrenocorticotrophic hormone (ACTH) secretion from the anterior pituitary. Glucocorticoids, synthesised in the adrenal cortex, act at multiple targets in the body to facilitate the necessary metabolic and behavioural adaptations required for stress coping and restoring physiological homeostasis. Moreover, glucocorticoids provide negative-feedback control of the HPA axis through actions on glucocorticoid and mineralocorticoid receptors in the brain and pituitary, crucial for terminating the stress response.

In addition to glucocorticoids, stress also results in a rapid increase in the secretion of other steroid hormones such as progesterone[9] and 11-deoxycorticosterone (DOC)[10] from the adrenal cortex. In the brain, these steroids can be further metabolised into neuroactive steroids, such as 5α-dihydroprogesterone (DHP) and 5α-dihydrodeoxycorticosterone (DHDOC) by 5α-reductase, which in turn may be converted into allopregnanolone and tetrahydrodeoxycorticosterone (THDOC), respectively, by 3αHSD (Figure 1), which are considered to fine-tune and aid cessation of the stress response. Indeed, levels of both allopregnanolone and THDOC are increased in the cerebral cortex and hypothalamus following exposure to acute stress[11] and both have been shown to negatively modulate stress-induced HPA axis activity in vivo.[12-15] Moreover, allopregnanolone prevents the up-regulation of CRH mRNA in the mpPVN induced by adrenalectomy, attenuates stimulated CRH release from hypothalamic explants and inhibits the firing of mpPVN CRH neurones in vitro[16,17], while administration of the 5α-reductase inhibitor, finasteride, leads to heightened HPA axis responses to stress in males and females.[13,18] Allopregnanolone, DHDOC and THDOC are positive allosteric modulators of $GABA_A$ receptors; by prolonging the opening time of chloride ion channels within $GABA_A$ receptors, they enhance the inhibitory actions of GABA.[19-22] Given that the PVN is substantially innervated by GABAergic neurones[23,24] and the majority of mpPVN CRH neurones express $GABA_A$ receptors,[25] this provides a means by which local neurosteroids may modulate inhibitory tone over the HPA axis.

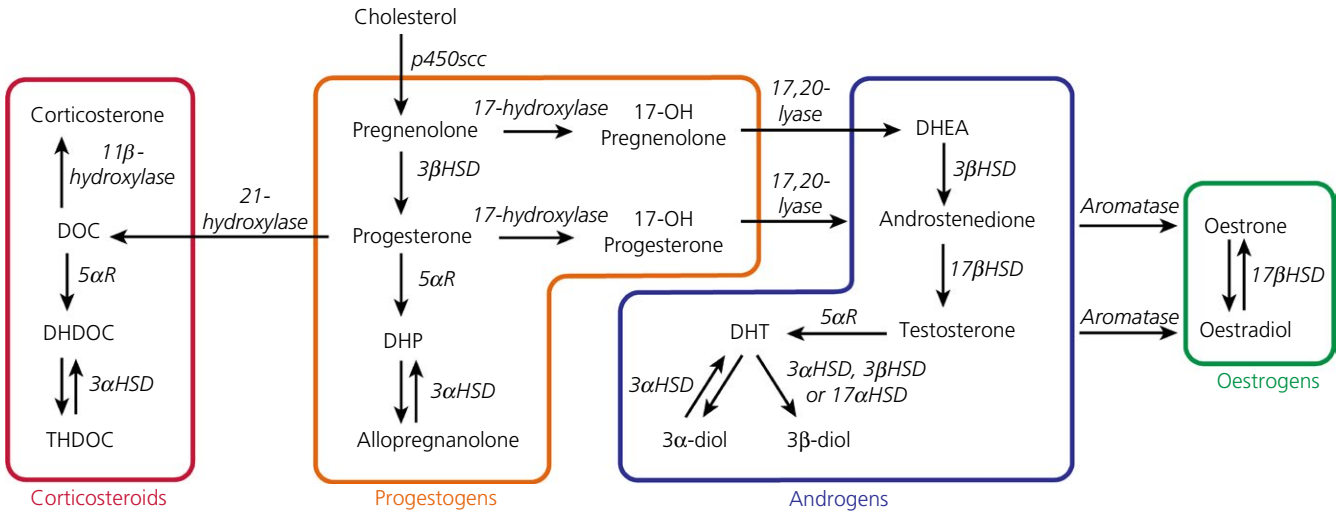

FIGURE 1 Biosynthetic pathways involved in steroidogenesis. The major pathways and enzymes involved in the biosynthesis of corticosteroids, progestogens, androgens and oestrogens from cholesterol. 3α-diol, 3α-androstanediol; 3αHSD, 3α-hydroxysteroid dehydrogenase (*Akr1c*); 3β-diol, 3β-androstanediol; 3-βHSD, 3β-hydroxysteroid dehydrogenase (*Hsd3b*); 5αR, 5α-reductase (*Srd5a*); 17αHSD, 17α-hydroxysteroid dehydrogenase (*Hsd17a*); 17βHSD, 17β-hydroxysteroid dehydrogenase (*Hsd17b*); DHDOC: 5α-dihydrodeoxycorticosterone; DHEA, dehydroepiandrosterone; DHP, 5α-dihydroprogesterone; DHT, 5α-dihydrotestosterone; DOC, 11-deoxycorticosterone; p450scc, cholesterol side-chain cleavage enzyme (*Cyp11a1*); THDOC, tetrahydrodeoxycorticosterone

It is well established that there are sex differences in both basal and stress-induced activity of the HPA axis in rodents. Females generally display greater concentrations of circulating corticosterone under nonstress conditions and this is particularly evident at the onset of the dark phase.[26,27] In addition, the HPA axis is more responsive to both physical and psychological stressors in females, than males, as reflected by greater secretion of ACTH and corticosterone and a larger increase in CRH gene transcription in the mpPVN.[27-31]

Intriguingly, stress-induced activation of neurones in limbic brain regions, such as the prefrontal cortex and hippocampus, is greater in males than in females.[32-34] Given that these regions project indirectly to the PVN (eg via a relay in the bed nucleus of the stria terminalis) and exert a net inhibitory effect on HPA axis activity,[35-37] lower stress responses in males may reflect greater inhibitory signalling from limbic brain regions to the PVN.

The sex differences in HPA axis activity have been largely attributed to gonadal steroids, given that oestradiol typically has stimulatory actions, whereas androgens have inhibitory effects on HPA axis function. For example, ovariectomy decreases,[38-40] whereas orchidectomy increases,[27,41-47] the HPA axis response to stress and these effects of gonadectomy can be normalised with testosterone[41,42,44,46] or oestradiol[38] replacement in males and females, respectively. However, the effect of testosterone on HPA axis activity is evidently mediated via the neuroactive metabolite of testosterone, 3β-androstandiol (3β-diol).[48] Likewise, studies indicate that progesterone may counteract some of the stimulatory effects of oestradiol on HPA axis activity,[49] although this is likely mediated via its 3α-hydroxy A ring-reduced neuroactive metabolite, allopregnanolone.[12,13] Despite these findings, it is not known whether there are sex differences in the stress-induced neuroactive steroid concentrations in the brain, which could potentially contribute to the differential HPA axis responses in males and females.

Although some studies have quantified the concentrations of a few steroids in the brain in response to foot-shock, forced swimming or CO_2 inhalation, previous work has focused on males and measurements have been largely limited to the cerebral cortex or hypothalamus.[11,50-52] In studies where male and females have been directly compared, this has been under nonstress conditions.[53,54] To date, no study has simultaneously measured stress-induced concentrations of multiple steroids in several different brain regions. Moreover, to the best of our knowledge, no study has compared changes in central steroid levels in response to stress in male and female rats in the same experiment. Traditional detection methods such as radioimmunoassays incur limitations: cross-reactivity issues may hinder accuracy, whereas low throughput and low sensitivity makes quantification of a panel of structurally related steroids in discrete brain regions difficult.[55,56]

Accordingly, in the present study, we used a liquid chromatography-mass spectrometry (LC-MS) method to quantify a panel of steroids in the plasma, as well as in five separate brain regions known to be involved in regulating the activity of the HPA axis, from male and female rats under nonstress and acute stress conditions. We aimed to establish: (i) which steroids (from our panel) are increased in the brain by acute stress; (ii) whether there are sex and/or regional differences in stress-induced central steroid concentrations; and (ii) whether circulating steroid concentrations correlate with those found in the brain following stress.

2 | MATERIALS AND METHODS

2.1 | Animals

Male and female Sprague-Dawley rats were bred in the rodent facility at the Roslin Institute. Rats were group housed by sex (four to six females, three or four males per cage) in open-top cages with ad libitum access to drinking water and a standard rodent diet (Harlan Teklad; Cambridgeshire, UK) and under a 12:12 hour light/dark cycle (lights on 8.00 AM) with controlled temperature (22±1˚C) and humidity (58±3%). Experiments were performed in rats aged 21 weeks and were approved by the local Animal Welfare and Ethical Review Body and performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and the European Directive (2010/63/EU).

2.2 | Stress paradigm

All experiments and tissue collection were performed in male and female (randomly cycling) rats between 10.00 AM and 12.30 PM. Forced swimming was used because: (i) it is a robust activator of the HPA axis[57,58]; (ii) sex differences in ACTH and corticosterone secretion have been demonstrated using this stressor[57]; and (iii) it is a combined physical and psychological stressor, and thus activates stress-responsive nuclei in both the forebrain and hindbrain.[59] For the stressed groups (n = 7 per group per sex), rats were placed in a glass cylinder (diameter 25 cm; height 50 cm) filled with water (21-22°C) to a depth of 30 cm and forced to swim for 2 minutes, after which they were gently dried in a towel and returned to their home cage. Thirty minutes after the onset of swimming stress, rats were rapidly transferred to a separate room and killed by conscious decapitation. This time-point has previously been shown to correspond with peak levels of immediate early gene induction[59] in the brain and elevated corticosterone secretion,[60] as well as with the peak in stress-induced allopregnanolone concentrations in the brain[11,50] following swim stress. For the control group, rats (n = 7 per group per sex) remained undisturbed in their home cage prior to killing (as before). In each case, trunk blood was collected in tubes containing 0.5 mL of ice-cold 5% (w/v) EDTA and plasma separated by centrifugation (1500 *g* at 4°C for 15 minutes). Brains were rapidly removed, and the regions of interest (frontal cortex, hypothalamus, amygdala, hippocampus, brainstem) were dissected (see Supporting information, Figure S1) and frozen on dry ice. Plasma was stored at −20°C and brain samples were stored at −80°C until further analysis.

2.3 | Steroid standards

All steroid standards were purchased from Steraloids Inc (Newport, RI, USA) and all solvents/chemicals used were high-performance

liquid chromatography (HPLC)-MS grade. Stock solutions of steroid standards (1 mg mL^{-1}) were prepared in methanol (Honeywell Riedel-de Haën, Seelze, Germany) and stored at −20°C. Steroids were combined and diluted in methanol into a working stock solution containing a concentration of 10 μg mL^{-1} of each steroid (except for allopregnanolone and progesterone with a concentration of 25 μg mL^{-1}). On the day of sample processing, standards were further diluted 100-fold in a surrogate matrix 4% (w/v) bovine serum albumin (BSA) (VWR, Leicester, UK) in PBS, followed by a serial 2.5-fold dilution in 4% BSA to produce seven calibrants. The calibration standards used ranged from 41 to 10 000 pg mL^{-1} for corticosterone, DOC (4-pregnen-21-ol-3,20-dione), DHDOC (5α-pregnan-21-ol-3,20-dione), testosterone, pregnenolone and DHP (5α-pregnane-3,20-dione), and 102.4 to 25 000 pg mL^{-1} for progesterone and allopregnanolone (5α-pregnan-3α-ol-20-one). The deuterated internal standard progesterone-d9 was also dissolved in methanol and diluted to a final concentration of 25 ng mL^{-1} in 50% methanol.

2.4 | Sample processing

Samples from all four groups from any given region were processed in the same run, together with seven standard calibrants and a zero sample containing only 4% BSA. For standard calibrants and plasma, 100 μL was used. Frozen brain regions were weighed prior to sample processing (n = 7 per group per sex, except for the frontal cortex, amygdala and hypothalamus where a sample from the female non-stressed group was lost).

2.4.1 | Tissue homogenisation

Brain samples were homogenised in 500 μL of methanol containing 1% formic acid (Fisher Scientific, Loughborough, UK). For plasma and standards, 400 μL of methanol containing 1% formic acid was added to 100 μL of plasma/standards and vigorously vortexed. Next, 20 μL of progesterone-d9 (25 ng mL^{-1}) was added and the homogenates were briefly sonicated. After incubation on dry ice for 30 minutes, the homogenates were centrifuged for 10 minutes (13 000 *g* at 4°C) and the supernatant was decanted into a borosilicate tube and the pellet re-extracted again with 500 μL of methanol containing 1% formic acid, and then sonicated and centrifuged as described above but without incubation. Supernatants were combined, then diluted with ultrapure water to a final concentration of 30% methanol (see Supporting information, Figure S2).

2.4.2 | Solid phase extraction

Steroids in both plasma and brain samples were extracted by solid phase extraction using DSC-Discovery C18 100 mg columns (#52602-U; Supelco, Belfont, PA, USA). Columns were activated with 1 mL of methanol and equilibrated with another 1 mL of 30% methanol. Diluted supernatants from homogenates (approximately 3 mL) were then loaded, followed by two 1-mL washes of 50% methanol. All steps were assisted by centrifugation at 50 *g* (average flow rate of 0.5 mL min^{-1}). Steroids were eluted with 1 mL of 85% methanol by gravity flow. The collected eluate was dried in a vacuum concentrator (Savant SpeedVac; ThermoFisher Scientific, Waltham, MA, USA) overnight (see Supporting information, Figure S2). Dried samples were stored at −20°C until the day of analysis.

2.4.3 | Derivatisation

On the day of LC-MS analysis, 400 μL of freshly prepared derivatisation agent (1 mg mL^{-1} of Girard's T reagent; #89397; Sigma-Aldrich, Gillingham, UK; dissolved in methanol containing 0.2% formic acid) was added to the dried samples. After incubation at 37°C for 30 minutes, the reaction was stopped by the addition of 50 μL of 5% ammonium hydroxide (ACROS Organics, Morris Plains, NJ, USA) in methanol. Samples were dried in the SpeedVac then reconstituted in 50 μL of 25 mmol L^{-1} phosphate buffer (pH 7.4) in 50% methanol to prevent acid hydrolysis of the derivatisation agent (see Supporting information, Figure S2). The sample was transferred to Chromacol vials (ThermoFisher Scientific) for analysis.

2.5 | LC-MS/MS analysis

Analysis of steroids was performed using an Ultimate 3000 Dionex HPLC system coupled to an AmaZon ETD ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). Separation of steroids on reverse phase HPLC was achieved on the ACE UltraCore 2.5 μm Super C18 column (75 mm by 2.1 mm inner diameter; Advance Chromatography Technologies, Aberdeen, UK) maintained at 40°C. Gradient elution was performed (see Supporting information, Table S1) with the mobile phase consisting of 50 mmol L^{-1} ammonium formate (Thermo Fisher Scientific), pH 3 (solvent A), and methanol with 0.1% formic acid (solvent B). Steroids were analysed simultaneously using multiple reaction monitoring (see Supporting information, Table S2), with positive electrospray ionisation and collision-induced fragmentation. Injections for all samples, including calibrants, were carried out in duplicate. The method allowed us to simultaneously determine the concentrations of eight steroids: corticosterone, DOC, DHDOC, pregnenolone, progesterone, DHP, allopregnanolone and testosterone (Figure 2) with a total runtime of 19 minutes (see Supporting information, Table S1). Because Girard's T reagent was used as the derivatisation agent, only steroids with a carbonyl group could be examined; thus, oestradiol and the testosterone metabolites, 3α- and 3β-androstanediol, which do not have this functional group, were not detected using this method. THDOC and DHT did not pass the validation criteria and thus these data are not included.

Data were acquired using HYSTAR software (Bruker Daltonics, Bremen, Germany) and peak area under the curve was extracted and automatically integrated using QUANTANALYSIS, version 2.0 (Bruker Daltonics). The ratio of the peak area of the target analyte and the peak area of progesterone-d9 was used to construct the calibration curve. The seven-point calibration curves were linear for all analytes (see Supporting information, Table S3). Concentrations of samples were extrapolated and converted to ng mL^{-1} (for plasma) or

normalised to the wet weight of the tissues (ng g^{-1}; for brain tissues). Performance characteristics (ie, recovery, sensitivity, accuracy and precision) were determined (see Supporting information, Table S4), and the concentrations of steroids detected in the plasma and brain were consistent with previous studies.[61,62]

2.6 | Statistical analysis

Data are presented as group means ± SEM. In each case, data were compared using a two-way ANOVA (SIGMAPLOT, version 11.0; Systat Software Inc., Chicago, IL, USA), with sex and stress as the two main factors followed by Student-Newman Keuls pairwise multiple comparison testing, except for the testosterone data, which were analysed using a two-tailed Student's *t*-test. The relationships between circulating and brain concentrations of steroids were determined using Pearson's correlation coefficients (SIGMAPLOT, version 11.0), and males and females were analysed separately. In each case, $P \leq 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Corticosterone

3.1.1 | Plasma

As expected, there was a main effect of stress ($F_{1,24} = 58.9$, $P < 0.001$) and sex ($F_{1,24} = 54.7$, $P < 0.001$) on corticosterone secretion and also a significant stress × sex interaction $F_{1,24} = 4.38$, $P = 0.047$). Plasma corticosterone concentrations were significantly greater in the stressed group compared to the control group in both sexes, with females displaying significantly greater plasma corticosterone concentrations compared to males under both stress and nonstress conditions (Figure 3).

3.1.2 | Brain

There were no significant differences detected in basal corticosterone concentrations between males and females in any of the brain regions (Figure 3). There were significant main effects of stress in each of the five brain regions examined (frontal cortex, $F_{1,23} = 50.6$, $P < 0.001$; hypothalamus, $F_{1,23} = 26.7$, $P < 0.001$; amygdala, $F_{1,23} = 14.8$, $P < 0.001$; hippocampus, $F_{1,24} = 45.4$, $P < 0.001$; brainstem, $F_{1,24} = 21.3$, $P < 0.001$), with corticosterone concentrations being significantly greater in the stressed rats compared to the nonstressed rats in both sexes (Figure 3). There was a significant main effect of sex observed only in the brainstem ($F_{1,24} = 5.30$, $P = 0.03$), where corticosterone concentrations were 1.8-fold greater in stressed females compared to stressed males.

3.1.3 | Correlations between central and circulating corticosterone

There were significant positive correlations between corticosterone concentrations in the plasma and in all brain regions, except the amygdala in males and the hypothalamus in females (Table 1).

3.2 | Deoxycorticosterone

3.2.1 | Plasma

There was no significant difference in plasma DOC concentrations between males and females under nonstress conditions; however,

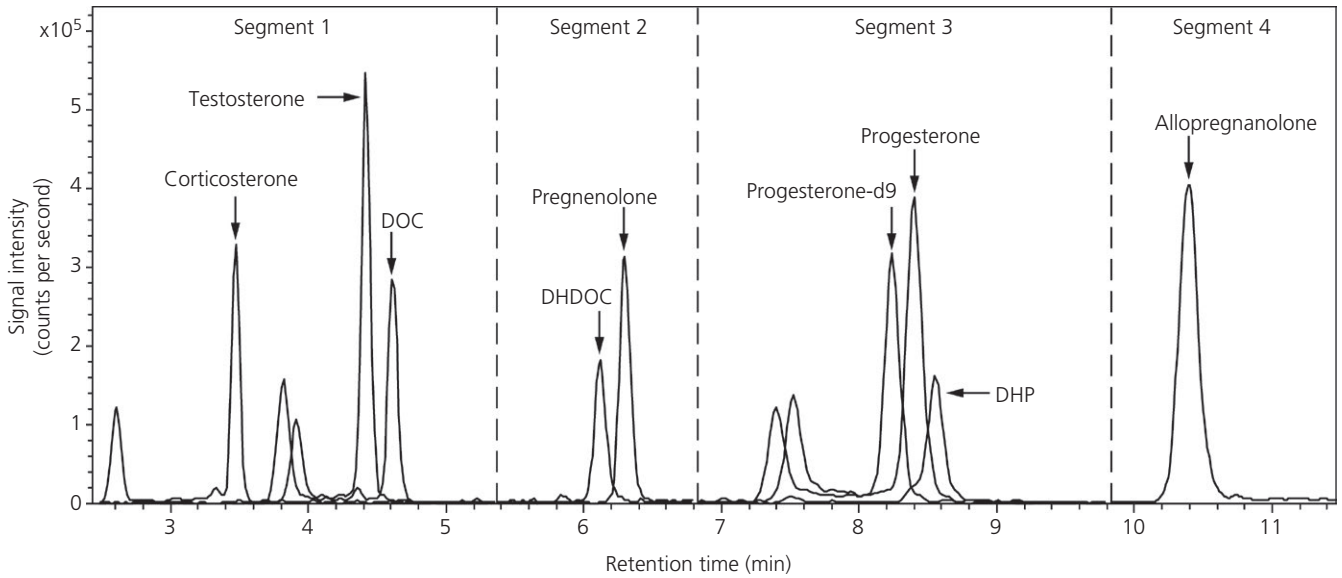

FIGURE 2 Representative chromatogram of the analytes. For some analytes, a double peak was observed as a result of the presence of both *syn* and *anti*-Girard's T derivatives, in which case the major product peak was used for quantification. The ratio between the major and minor peak was always consistent between runs. DHDOC: 5α-dihydrodeoxycorticosterone; DHP, 5α-dihydroprogesterone; DOC, 11-deoxycorticosterone

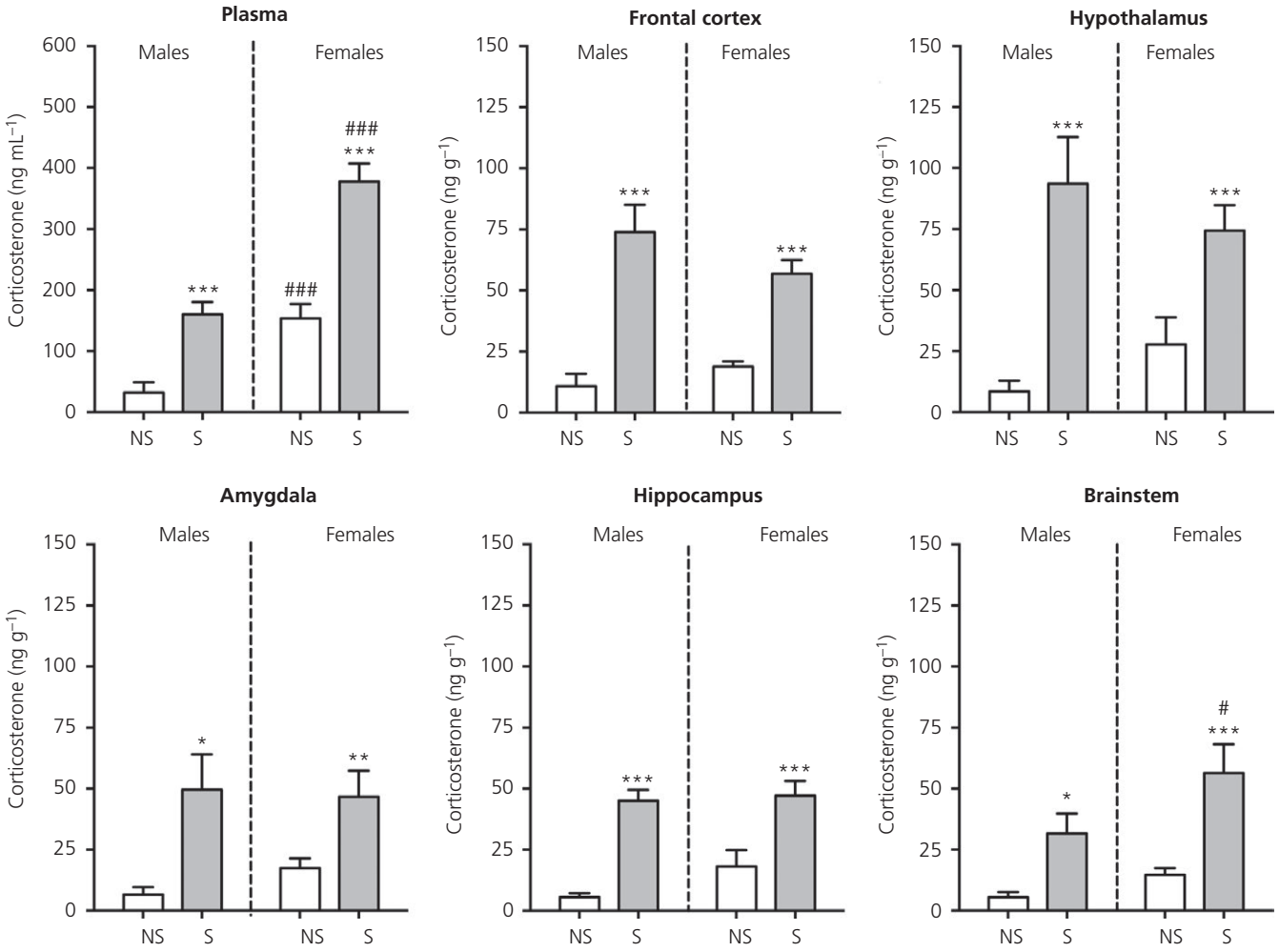

FIGURE 3 Effect of swim stress on corticosterone concentrations in the plasma and brain. Asterisks denote significant differences in the stressed group (S) compared to the nonstressed group (NS) within the same sex (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$); hashes denote significant sex differences within the same stress status ($^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$). Following stress, sex differences were detected for corticosterone concentrations in the plasma and brainstem. n = 6-7 rats per group. Note the difference in the scale of the *y*-axis for plasma

there was a significant effect of stress ($F_{1,24} = 67.6$, $P < 0.001$) and sex ($F_{1,24} = 26.0$, $P < 0.001$) and a significant stress × sex interaction ($F_{1,24} = 20.7$, $P < 0.001$). Stress resulted in significantly greater circulating DOC concentrations in both sexes (Figure 4). However, under stressed conditions, females exhibited significantly greater plasma DOC concentrations compared to males (Figure 4).

3.2.2 | Brain

There were no differences in basal concentrations of DOC between males and females in any of the regions studied (Figure 4). There were significant main effects of stress in all five brain regions (frontal cortex: $F_{1,23} = 72.0$, $P < 0.001$; hypothalamus: $F_{1,23} = 37.1$, $P < 0.001$; amygdala: $F_{1,23} = 56.7$, $P < 0.001$; hippocampus: $F_{1,24} = 103.1$, $P < 0.001$; brainstem: $F_{1,24} = 105.1$, $P < 0.001$). Central DOC concentrations were significantly greater in the stressed groups compared to the basal groups for both sexes (Figure 4). There were additional main effects of sex observed for the hippocampus ($F_{1,24} = 5.34$, $P = 0.03$) and brainstem ($F_{1,24} = 14.4$, $P < 0.001$) and a stress × sex interaction in the brainstem ($F_{1,24} = 12.9$, $P = 0.001$). Comparing the stressed groups, females had significantly greater DOC concentrations in the hippocampus and brainstem compared to males but not in the frontal cortex, hypothalamus or amygdala (Figure 4).

3.2.3 | Correlations between central and circulating DOC

There were significant positive correlations between DOC concentrations in the plasma and all five brain regions for both sexes (Table 1).

3.3 | Dihydrodeoxycorticosterone

3.3.1 | Plasma

There were no significant differences in plasma DHDOC concentrations in any of the groups (Figure 5).

TABLE 1 Correlation between central and circulating steroids

		Males		Females	
Steroid	**Region**	***r***	***P* value**	***R***	***P* value**
Corticosterone	Frontal cortex	0.89	<0.001***	0.88	<0.001***
	Hypothalamus	0.81	<0.001***	0.55	0.051*
	Amygdala	0.51	0.062	0.73	0.005**
	Hippocampus	0.82	<0.001***	0.62	0.017*
	Brainstem	0.62	0.019*	0.70	0.006**
DOC	Frontal cortex	0.80	0.001***	0.90	<0.001***
	Hypothalamus	0.76	0.002**	0.75	0.003**
	Amygdala	0.75	0.002**	0.85	<0.001***
	Hippocampus	0.87	<0.001***	0.88	<0.001***
	Brainstem	0.84	<0.001***	0.88	<0.001***
DHDOC	Frontal cortex	−0.07	0.826	0.23	0.459
	Hypothalamus	0.04	0.888	0.60	0.032*
	Amygdala	0.80	0.001***	0.92	<0.001***
	Hippocampus	0.19	0.525	0.68	0.008**
	Brainstem	−0.23	0.427	−0.16	0.591
Pregnenolone	Frontal cortex	−0.010	0.738	−0.02	0.942
	Hypothalamus	0.14	0.631	0.40	0.170
	Amygdala	0.53	0.051*	0.56	0.045*
	Hippocampus	0.06	0.828	0.48	0.079
	Brainstem	−0.25	0.388	−0.15	0.619
Progesterone	Frontal cortex	0.94	<0.001***	0.90	<0.001***
	Hypothalamus	0.98	<0.001***	0.82	0.001***
	Amygdala	0.99	<0.001***	0.83	<0.001***
	Hippocampus	0.94	<0.001***	0.89	<0.001***
	Brainstem	0.93	<0.001***	0.88	<0.001***
DHP	Frontal cortex	0.17	0.571	0.18	0.552
	Hypothalamus	−0.28	0.342	0.50	0.086
	Amygdala	0.83	<0.001***	0.94	<0.001***
	Hippocampus	0.01	0.747	0.66	0.011*
	Brainstem	−0.20	0.491	0.11	0.710
Allopregnanolone	Frontal cortex	−0.03	0.918	−0.02	0.952
	Hypothalamus	0.04	0.907	0.35	0.246
	Amygdala	0.59	0.026*	0.54	0.055
	Hippocampus	0.43	0.127	0.35	0.227
	Brainstem	0.41	0.143	−0.11	0.710
Testosterone	Frontal cortex	0.81	<0.001***		
	Hypothalamus	0.89	<0.001***		
	Amygdala	0.47	0.094		
	Hippocampus	0.49	0.075		
	Brainstem	0.80	0.001***		

Pearson's correlation coefficient (*r*) and probability (*P*) values between plasma and brain concentrations of steroids. Males and females were analysed separately and data from both stressed and nonstressed groups within the same sex were used for analysis (n = 13-14 per sex).
Asterisks denote significant correlations with plasma concentrations (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

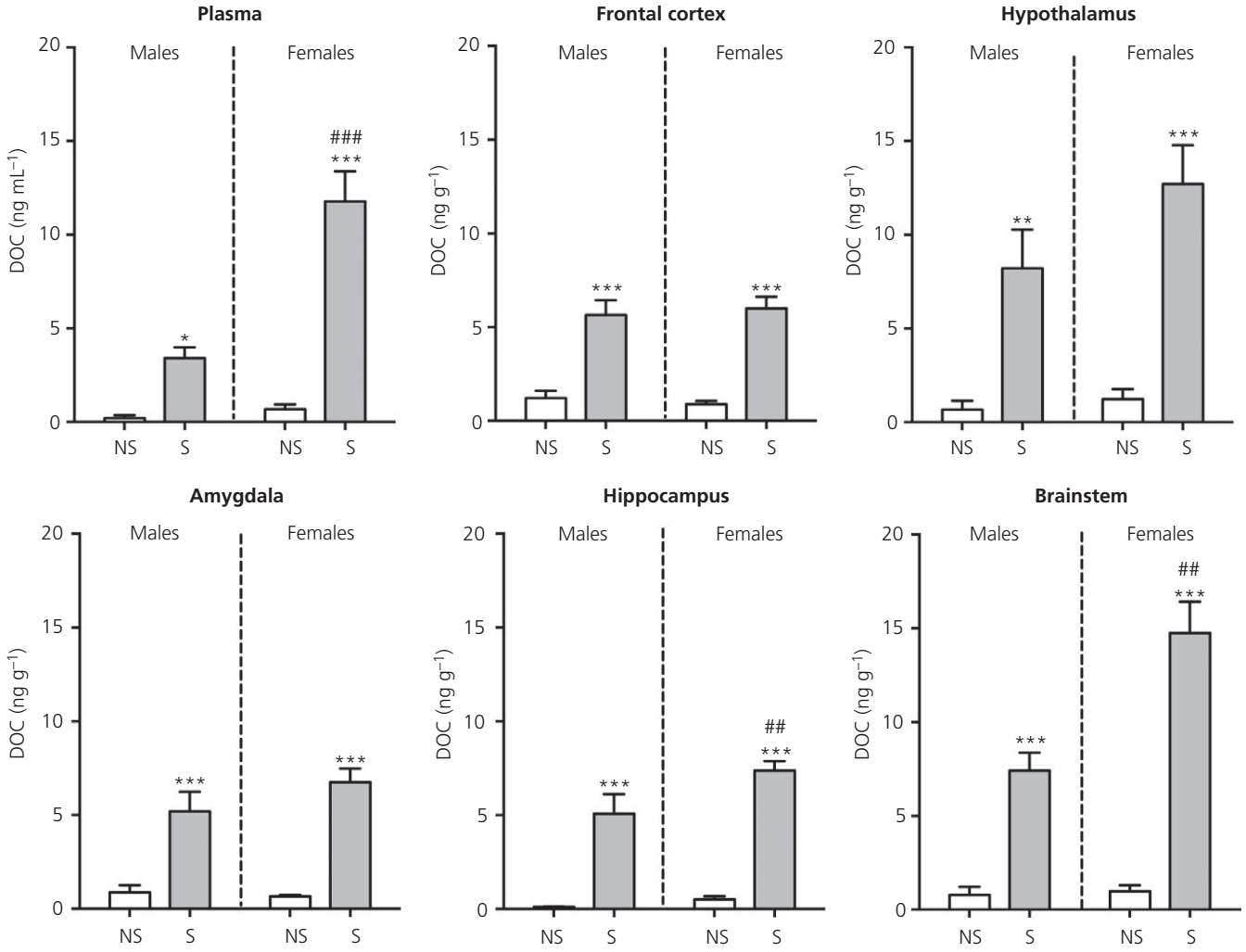

FIGURE 4 Effect of swim stress on DOC concentrations in the plasma and brain. Asterisks denote significant differences in the stressed group (S) compared to the nonstressed group (NS) within the same sex (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$); hashes denote significant sex differences with the same stress status ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$). After stress exposure, sex differences were detected for DOC concentrations in the plasma, hippocampus and brainstem. n = 6-7 rats per group

3.3.2 | Brain

There were significant main effects of stress in all brain regions, except for the amygdala (frontal cortex, $F_{1,23} = 16.1$, $P < 0.001$; hypothalamus, $F_{1,23} = 8.46$, $P = 0.008$; hippocampus, $F_{1,24} = 7.67$, $P = 0.01$; brainstem, $F_{1,24} = 5.68$, $P = 0.025$) (Figure 5). There were significant main effects of sex on DHDOC concentrations in the frontal cortex ($F_{1,23} = 6.09$, $P = 0.02$) and brainstem ($F_{1,24} = 4.35$, $P = 0.048$). In the frontal cortex, DHDOC concentrations were greater in both the stressed males and females compared to their respective control groups, with stress-induced concentrations being significantly greater in females compared to males. However, in the hypothalamus, hippocampus and brainstem, stress resulted in greater DHDOC concentrations only in females.

3.3.3 | Correlations between central and circulating DHDOC

In males, only the DHDOC concentration in the amygdala was correlated with plasma levels, whereas, in females, there were positive correlations between DHDOC concentrations in the plasma and those in the amygdala, hippocampus and hypothalamus (Table 1).

3.4 | Pregnenolone

3.4.1 | Plasma

There were no significant differences in plasma pregnenolone concentrations between any of the groups (Figure 6).

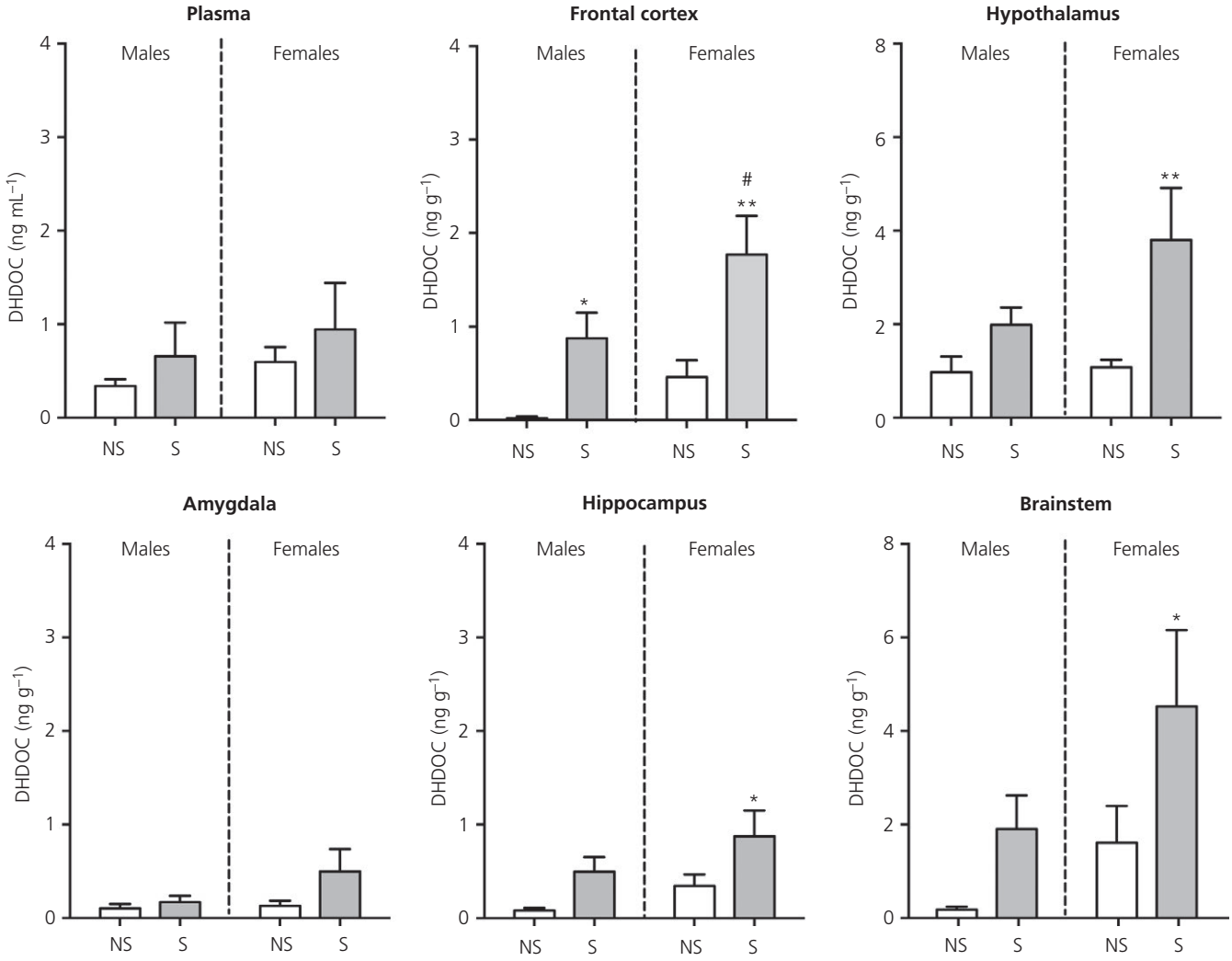

FIGURE 5 Effect of swim stress on DHDOC concentrations in the plasma and brain. Asterisks denote significant differences in the stressed group (S) compared to the nonstressed group (NS) within the same sex (*$P < 0.05$; **$P < 0.01$); hashes denote significant sex differences within the same stress status ($^{\#}P < 0.05$). Following stress, there was a significant sex difference in the frontal cortex and a tendency towards greater DHDOC concentrations in the brainstem of stressed females compared to stressed males ($P = 0.068$). n = 6-7 rats per group. Note the difference in the scaling of the *y*-axis for the hypothalamus and brainstem

3.4.2 | Brain

There was a significant main effect of sex (frontal cortex, $F_{1,23} = 5.44$, $P = 0.029$; hypothalamus, $F_{1,23} = 7.75$, $P = 0.01$; amygdala, $F_{1,23} = 20.2$, $P < 0.001$; hippocampus $F_{1,24} = 27.3$, $P < 0.001$; brainstem, $F_{1,24} = 19.1$) and stress (frontal cortex, $F_{1,23} = 39.2$, $P < 0.001$; hypothalamus, $F_{1,23} = 10.4$, $P = 0.004$; amygdala, $F_{1,23} = 40.9$, $P < 0.001$; hippocampus; $F_{1,24} = 39.9$, $P < 0.001$; brainstem, $F_{1,24} = 26.7$) in all of the brain regions examined (Figure 6). There was also a significant sex × stress interaction in the brainstem ($F_{1,24} = 4.89$), amygdala ($F_{1,23} = 12.8$, $P = 0.002$) and hippocampus ($F_{1,24} = 7.25$, $P = 0.01$). There was no difference in basal levels of pregnenolone in the brain between the sexes (Figure 6). Following stress, both males and females had greater pregnenolone concentrations than their respective control groups in all brain regions, except for the hypothalamus, where only females showed significantly greater pregnenolone concentrations compared to nonstressed females. For each brain region examined, stress-induced pregnenolone concentrations were greater in females versus males (Figure 6).

3.4.3 | Correlations between central and circulating pregnenolone

For both males and females, the only significant correlation observed with plasma concentrations of pregnenolone was in the amygdala (Table 1). No other significant correlations were detected between circulating and brain pregnenolone concentrations.

3.5 | Progesterone

3.5.1 | Plasma

There were significant main effects of stress ($F_{1,24} = 49.1$, $P < 0.001$) and sex ($F_{1,24} = 49.7$, $P < 0.001$) and also a significant stress × sex

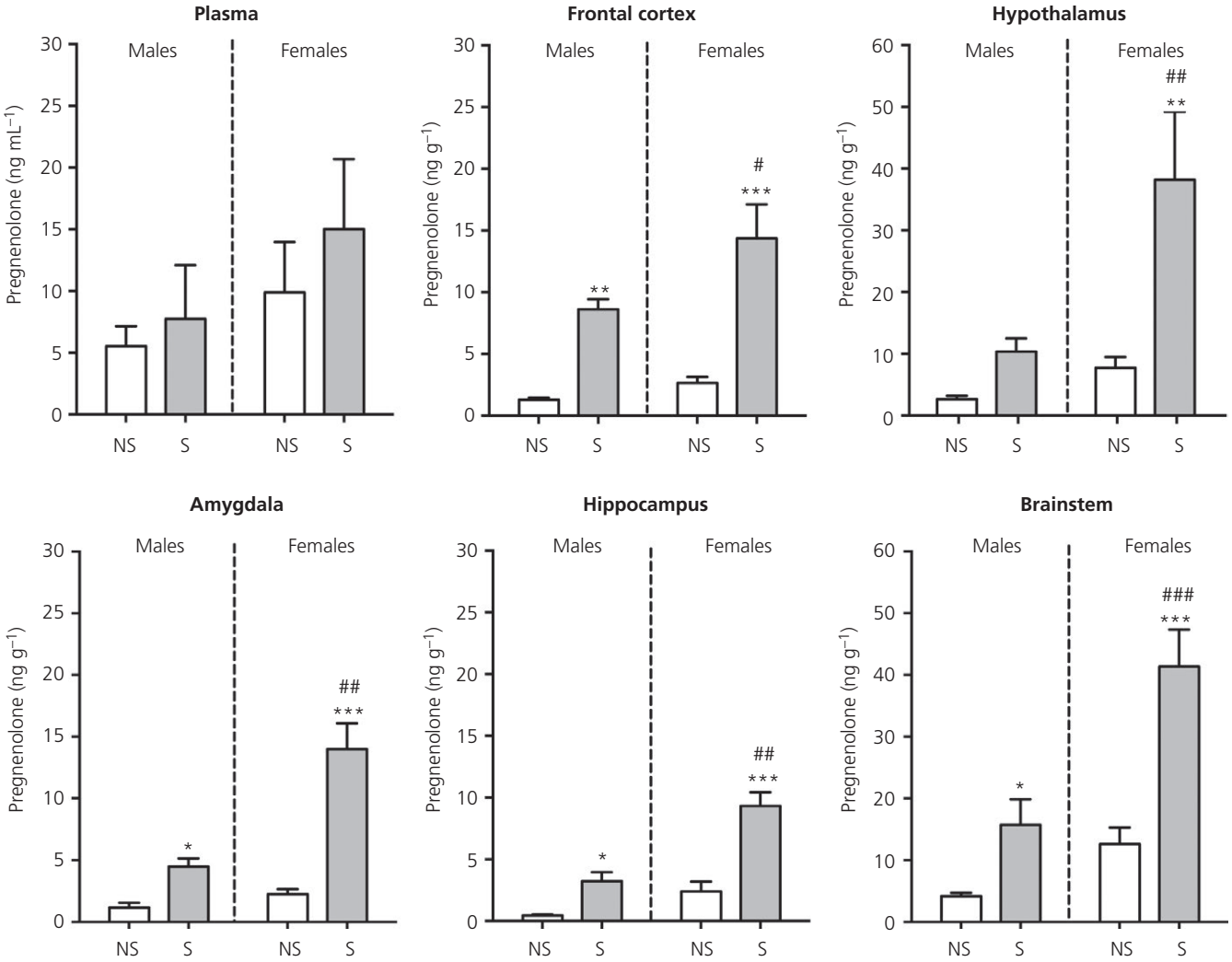

FIGURE 6 Effect of swim stress on pregnenolone concentrations in the plasma and brain. Asterisks denote significant differences in the stressed group (S) compared to the nonstressed group (NS) within the same sex (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$); hashes denote significant sex differences with the same stress status ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$). Concentrations of pregnenolone were greater in stressed females vs the stressed males in all of the brain regions examined. n = 6-7 rats per group. Note the difference in the scaling of the *y*-axis for the hypothalamus and brainstem

interaction ($F_{1,24} = 10.8$, $P = 0.003$) in plasma progesterone concentrations. Under basal conditions, circulating progesterone was significantly greater in females compared to males (Figure 7). In both sexes, stress resulted in increased progesterone secretion, with greater circulating concentrations in females than in males (Figure 7).

3.5.2 | Brain

There were significant main effects of stress and sex in all five brain regions examined (frontal cortex: stress $F_{1,23} = 62.6$, $P < 0.001$ and sex $F_{1,23} = 13.6$, $P = 0.001$; hypothalamus: stress $F_{1,23} = 48.3$, $P < 0.001$ and sex $F_{1,23} = 28.7$, $P < 0.001$; amygdala: stress $F_{1,23} = 50.7$, $P < 0.001$ and sex $F_{1,23} = 23.0$, $P < 0.001$; hippocampus: stress $F_{1,24} = 53.9$, $P < 0.001$ and sex $F_{1,24} = 38.9$, $P < 0.001$; brainstem: stress $F_{1,24} = 31.9$ and sex $F_{1,24} = 18.8$). There were also stress × sex interactions observed for the hypothalamus ($F_{1,23} = 8.14$, $P = 0.009$), amygdala ($F_{1,23} = 4.48$, $P = 0.045$) and hippocampus ($F_{1,24} = 3.94$, $P = 0.058$). There was a tendency for higher basal progesterone concentrations in females compared to males; however, this was only statistically significant in the hippocampus (Figure 7). Following stress, progesterone concentrations in all five brain regions were significantly higher than those measured under basal conditions in both sexes. Moreover, in each case, stress-induced progesterone concentrations were greater in females than in males (Figure 7).

3.5.3 | Correlations between central and circulating progesterone

There were significant positive correlations between circulating progesterone concentrations and those measured in all five brain regions for both males and females (Table 1).

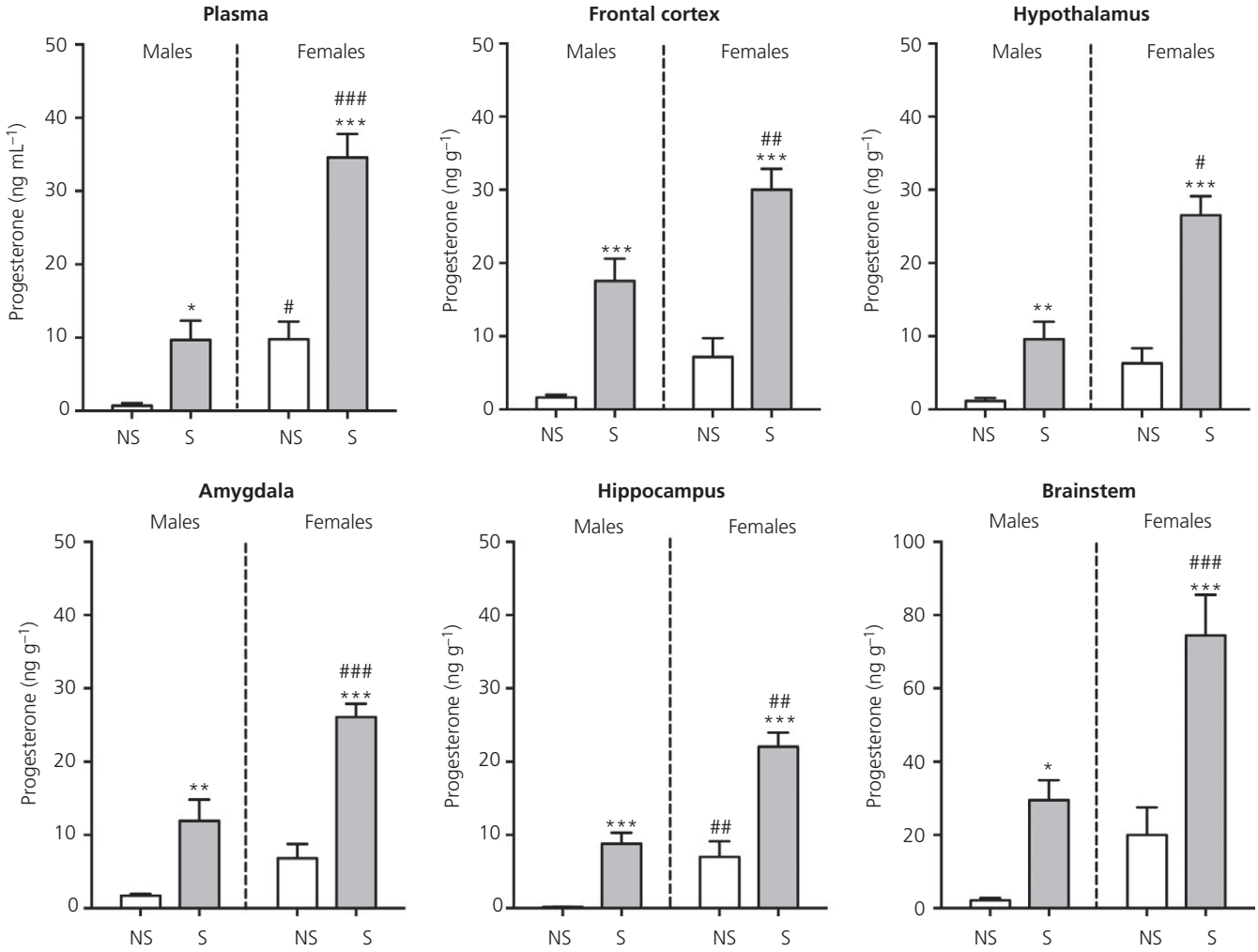

FIGURE 7 Effect of swim stress on progesterone concentrations in the plasma and brain. Asterisks denote significant differences in the stressed group (S) compared to the nonstressed group (NS) within the same sex (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$); hashes denote significant sex differences within the same stress status ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$). Following stress, sex differences in progesterone concentrations were detected in the plasma and all regions of the brain. n = 6-7 rats per group. Note the difference in the scaling of the *y*-axis for the brainstem

3.6 | Dihydroprogesterone

3.6.1 | Plasma

There was no significant difference in circulating DHP in any of the groups investigated (Figure 8).

3.6.2 | Brain

There was a significant main effect of stress on DHP concentration in the frontal cortex ($F_{1,23} = 13.1$, $P = 0.001$), hypothalamus ($F_{1,23} = 7.14$, $P = 0.01$), amygdala ($F_{1,23} = 5.73$, $P = 0.03$), hippocampus ($F_{1,24} = 9.77$, $P = 0.005$) and brainstem ($F_{1,24} = 22.0$, $P < 0.001$), as well as a main effect of sex for the hypothalamus ($F_{1,23} = 24.0$, $P < 0.001$) and brainstem ($F_{1,24} = 16.0$, $P < 0.001$). Under basal conditions, DHP concentrations in the hypothalamus were significantly greater in females than in males and there was a similar trend observed for the brainstem ($P = 0.06$) (Figure 8). Following stress, females had significantly higher DHP in each of the brain regions investigated compared to nonstress conditions, whereas, in males, stress-induced concentrations of DHP were greater than basal levels only in the frontal cortex and brainstem (Figure 8). There was a sex difference in stress-induced DHP concentrations, with stressed females exhibiting greater DHP than stressed males in the amygdala, hypothalamus and brainstem (Figure 8).

3.6.3 | Correlations between central and circulating DHP

There were significant positive correlations observed between DHP concentrations in the amygdala and circulation in both males and females (Table 1). In females, there was also a modest correlation between the DHP concentrations in the plasma and the hippocampus (Table 1). No other correlations were detected between central and circulating DHP levels.

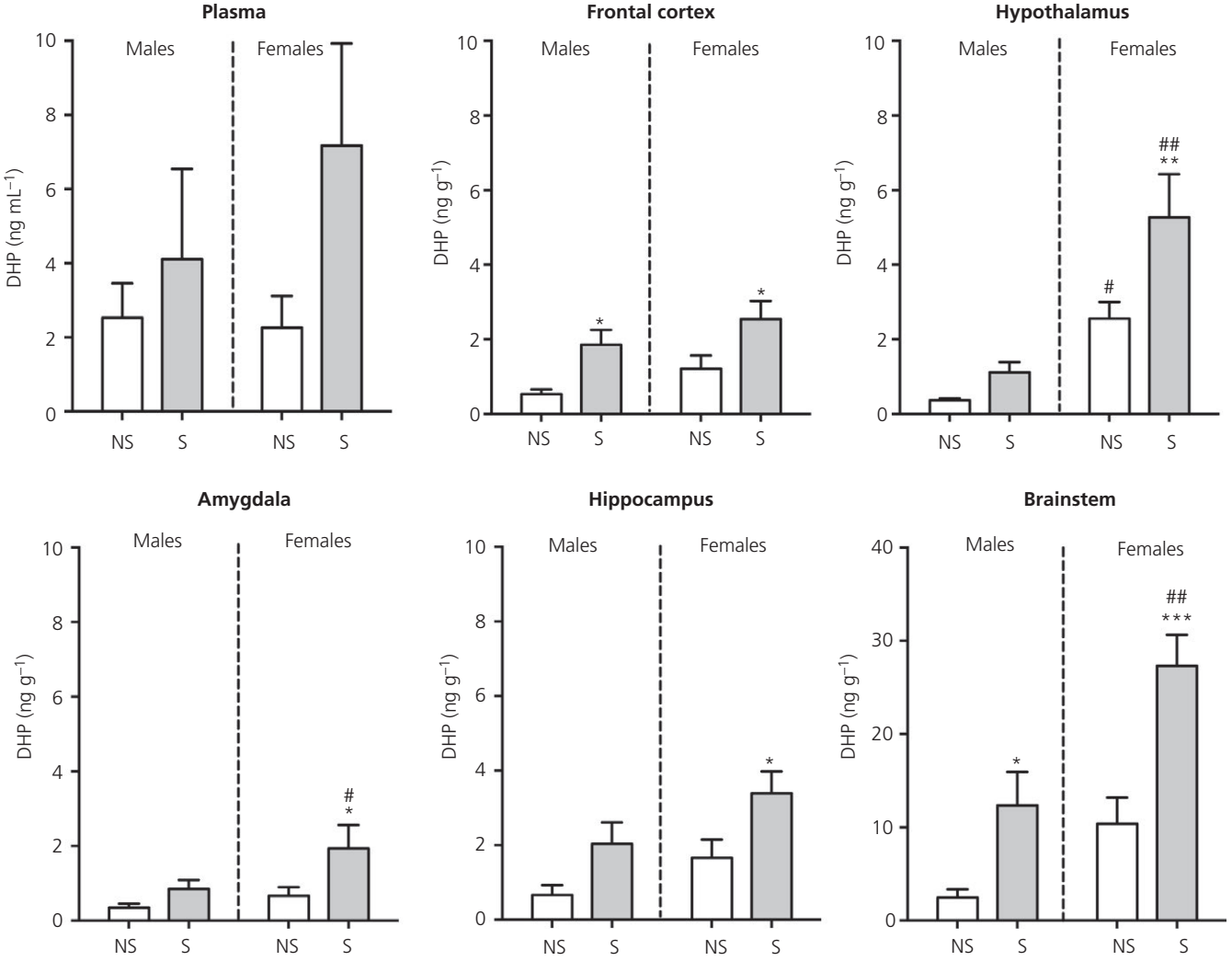

FIGURE 8 Effect of swim stress on DHP concentrations in the plasma and brain. Asterisks denote significant differences in the stressed group (S) compared to the nonstressed group (NS) within the same sex (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$); hashes denote significant sex differences within the same stress status ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$). Under nonstress conditions, DHP concentrations were greater in the hypothalamus of females compared to males and a similar trend was detected in the brainstem ($P = 0.06$). Following stress, significant sex differences in DHP concentrations were detected in the hypothalamus, amygdala and brainstem. n = 6-7 rats per group. Note the difference in the scaling of the *y*-axis for the brainstem

3.7 | Allopregnanolone

3.7.1 | Plasma

There was an effect of sex on circulating allopregnanolone concentrations ($F_{1,24} = 21.9$, $P < 0.001$). Basal allopregnanolone concentrations were significantly greater in females than in males (Figure 9). There was no main effect of stress on plasma allopregnanolone concentrations in either sex (Figure 9).

3.7.2 | Brain

There were significant main effects of sex on allopregnanolone concentrations for each of the brain regions examined (frontal cortex, $F_{1,23} = 14.9$, $P < 0.001$; hypothalamus, $F_{1,23} = 13.1$, $P = 0.001$; amygdala, $F_{1,23} = 80.2$, $P < 0.001$; hippocampus, $F_{1,24} = 20.9$, $P < 0.001$; brainstem, $F_{1,24} = 40.2$, $P < 0.001$). A significant main effect of stress was observed for allopregnanolone concentrations in the frontal cortex ($F_{1,23} = 5.71$, $P = 0.03$), amygdala ($F_{1,23} = 27.6$, $P < 0.001$) and brainstem ($F_{1,24} = 9.08$, $P = 0.006$). A stress × sex interaction was additionally observed in the amygdala ($F_{1,23} = 14.8$, $P < 0.001$). Basal allopregnanolone concentrations were significantly greater in females than in males in all of the brain regions, except the hypothalamus, where a tendency for higher levels was observed ($P = 0.07$) (Figure 9). Stress resulted in significantly greater allopregnanolone concentrations in the frontal cortex, amygdala and brainstem compared to nonstressed females (Figure 9). Similar trends were observed in the male brain, although this only reached statistical significance for the frontal cortex.

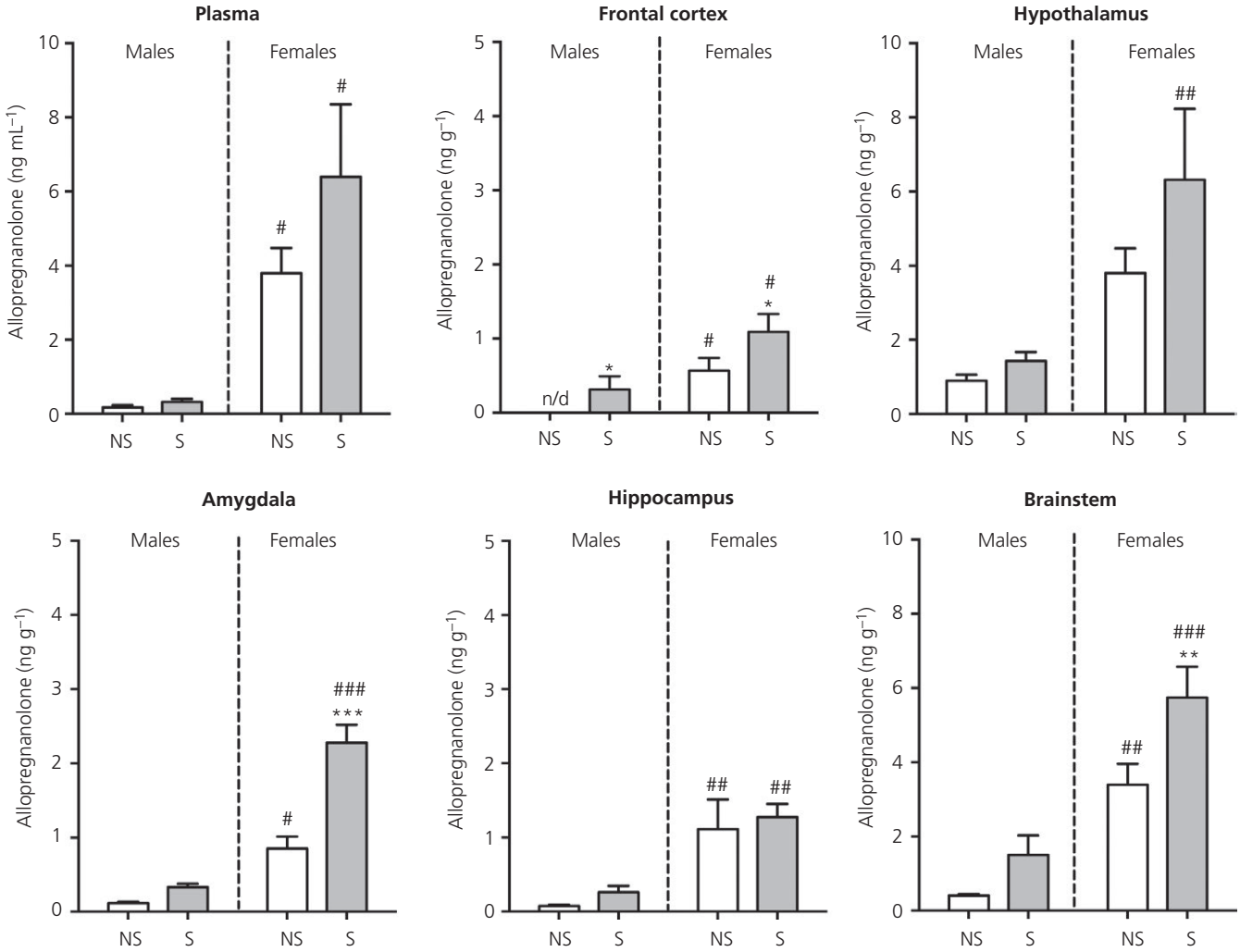

FIGURE 9 Effect of swim stress on allopregnanolone concentrations in the plasma and brain. Asterisks denote significant differences in the stressed group (S) compared to the nonstressed group (NS) within the same sex (*P < 0.05; **P < 0.01; ***P < 0.001); hashes denote significant sex differences with the same stress status ($^{\#}P$ < 0.05; $^{\#\#}P$ < 0.01; $^{\#\#\#}P$ < 0.001). Under nonstress conditions, allopregnanolone concentrations were greater in females compared to males in the plasma and each of the brain regions examined, except the hypothalamus where there was a tendency for higher levels in females (P = 0.07). After stress exposure, significant sex differences in allopregnanolone concentrations were detected in the plasma and all regions of the brain. n/d, not detected. n = 6-7 rats/group. Note the difference in the scaling of the y-axis for the plasma, hypothalamus and brainstem

3.7.3 | Correlations between central and circulating allopregnanolone

In both sexes, only allopregnanolone concentrations in the amygdala were found to be significantly correlated with circulating allopregnanolone (Table 1).

3.8 | Testosterone

3.8.1 | Plasma

There was no significant effect of stress on plasma testosterone concentrations in males (1.05 ± 0.28 ng mL^{-1} in nonstressed rats vs 0.77 ± 0.20 ng mL^{-1} in stressed rats). Testosterone was below the lower limit of quantification (ie <41 pg mL^{-1}) in the plasma from females (see Supporting information, Table S4).

3.8.2 | Brain

Testosterone was below the lower limit of quantification in all of the brain regions in females. There was no significant effect of stress on central testosterone concentrations in male rats (frontal cortex, 2.20 ± 0.45 vs 2.20 ± 0.58 ng g^{-1}; hypothalamus, 2.79 ± 0.62 vs 3.15 ± 1.23 ng g^{-1}; amygdala, 0.88 ± 0.17 vs 1.03 ± 0.24 ng g^{-1}; hippocampus, 1.17 ± 0.21 vs 1.71 ± 0.61 ng g^{-1}; brainstem, 2.03 ± 0.19 vs 2.90 ± 0.35 ng g^{-1} in the stressed and nonstressed groups, respectively).

3.8.3 | Correlations between central and circulating testosterone

Concentrations of testosterone in the frontal cortex, amygdala, hypothalamus and brainstem were positively correlated with circulating testosterone in male rats (Table 1).

4 | DISCUSSION

In the present study, we investigated the impact of an acute stressor on the circulating and central steroid profile in male and female rats. Several key findings emerged in the present study. We show for the first time there are clear sex differences in the brain's steroid response to acute swim stress in the rat, with females typically showing markedly greater concentrations in most cases. We also report that, although concentrations of corticosterone, progesterone and DOC in the brain are positively correlated with those measured in the circulation, this is not the case for pregnenolone, DHP, DHDOC and allopregnanolone, supporting the concept of the de novo synthesis of these neurosteroids in the brain in response to stress.[7,11] Lastly, although the effect of stress on the steroidal milieu of the brain was largely global, we did find evidence for regional differences (Figure 10), particularly for DHDOC and allopregnanolone, which may be of functional relevance.

A key feature of the endocrine response to stress is increased secretion of corticosterone from the adrenal cortex into the bloodstream. It is well established that females display enhanced HPA axis activity, characterised by a greater circulating concentrations of corticosterone under basal conditions and a larger increase in ACTH and corticosterone secretion following acute stress.[63-65] Consistent with this, females in the present study exhibited greater basal and stress-induced plasma corticosterone concentrations. In the periphery, greater corticosterone secretion in females is associated with a

FIGURE 10 Summary of the key changes in steroid concentrations induced by stress. Changes in steroid concentrations in the plasma and brain 30 minutes after a forced swim stress. The source of steroids in the plasma is largely from peripheral steroidogenic organs, such as the adrenal glands and gonads, whereas steroids in the brain may be peripherally or centrally derived. Blue and red arrows represent stress-induced increases in steroid concentrations in males and females, respectively. Dashes indicate no significant changes following stress. Hashes denote the presence of a sex difference under stressed conditions. AP, allopregnanolone; CORT, corticosterone; DHDOC, dihydrodeoxycorticosterone; DHP, dihydroprogesterone; DOC, deoxycorticosterone; PREG, pregnenolone; P4, progesterone; T, testosterone

heightened sensitivity of the anterior pituitary to CRH and the adrenal gland to ACTH, as well as a greater capacity for glucocorticoid synthesis in the adrenal glands,[66] probably as a result of the actions of oestradiol.[39] In the brain, CRH mRNA expression in the mpPVN is increased following stress to a greater extent in females than in males,[31] which may be further compounded by diminished GR-mediated negative-feedback control of the HPA axis in females.[67] Interestingly, despite sex differences in the plasma, corticosterone concentrations in the female brain were not different from those in males, except for in the brainstem. This is consistent with the finding of similar circadian corticosterone rhythms and concentrations in the brains of males and females, despite differences in the plasma.[26,27,63,68] It is likely that greater concentrations of corticosteroid-binding globulin (which restricts the amount of free corticosterone in the blood that can enter the brain) in females[64,69] contribute to this finding. It is not known whether sex differences exist in the expression of 11β-hydroxysteroid dehydrogenase type 1 (which reactivates corticosterone from its inert metabolite, 11-dehydrocorticosterone) in the brain, which could play a role in modulating the local concentrations of corticosterone in the brain. However, sex and region-specific differences in 11β-hydroxylase (Cyp11b1) expression have been reported in the brain,[70] suggesting that differences in local corticosterone synthesis could also potentially contribute.

Under nonstress conditions, circulating progesterone concentrations were greater in females than males, which is consistent with previous findings[53,71]; however, there was no sex difference in plasma DOC concentrations. Following stress, circulating concentrations of DOC and progesterone were increased in both sexes, with significantly greater responses in females. Stress also resulted in greater levels of progesterone and DOC in both sexes in each of the brain regions investigated. Despite evidence of low levels of 21-hydroxylase gene expression and activity in the rodent brain,[72,73] DOC is considered to be primarily synthesised from progesterone in the adrenal cortex and is secreted from the adrenal glands, together with progesterone, in response to stress.[9,10] Consistent with this was our finding of a positive correlation between circulating DOC and progesterone concentrations and those in the brain, indicating that the likely source of DOC and progesterone in the brain is from the periphery, specifically adrenal derived. Although there was a robust sex difference in circulating DOC after stress, centrally, this was reflected only in the brainstem. This may be a result of sex differences in DOC metabolism in limbic brain regions in females or that circulating DOC more readily accesses the brainstem.

Importantly, DOC and progesterone mediate rapid nongenomic effects indirectly via their downstream neuroactive metabolites THDOC and allopregnanolone (Figure 1), which act as allosteric modulators of $GABA_A$ receptor activity[1,74] to enhance inhibitory GABA neurotransmission. THDOC and allopregnanolone are synthesised from DOC and progesterone via the intermediates DHDOC and DHP, respectively, and their conversion is catalysed by the same enzymes: 5α-reductase and 3αHSD (Figure 1). In the present study, stress resulted in higher concentrations of both DHDOC and DHP in discrete brain regions but not in the plasma, suggesting de novo synthesis of these steroids in the brain. Moreover, differential regulation was observed depending on sex and brain region. For example, DHDOC concentrations were found to be increased only in the frontal cortex of males following stress, whereas, for females, DHDOC concentrations were increased in the frontal cortex, hypothalamus, hippocampus and brainstem. Similarly, although stress increased DHP concentrations in each of the five brain regions examined in females, in males, stress-induced elevations in DHP were only detected in the frontal cortex and brainstem. In addition to the presence of greater levels of precursors in the female brain, differential expression of the 5α-reductase may contribute to the sex difference, given that expression has been shown to be higher in females, at least in the hippocampus.[75] Moreover, regional differences in expression and activity have been demonstrated for 5α-reductase and 3α-HSD in the adult brain,[76-79] with sexual dimorphic expression being displayed after stress exposure.[80] Any direct effects of DHDOC and DHP are unclear, although DHP is known to have affinity for the progesterone receptor[81] and DHDOC can potentiate GABA-activated chloride ion currents in neurones,[22] suggesting that, similar to THDOC and allopregnanolone, DHDOC also acts as a positive allosteric modulator at $GABA_A$ receptors.

Roles for allopregnanolone and THDOC in regulating HPA axis activity in response to stress via potentiating the inhibitory effects of GABA are well established.[12-14,16,74,82,83] Unfortunately, we were unable to reliably quantify THDOC using our method; however, we were able to measure allopregnanolone. Under nonstress conditions, circulating and central allopregnanolone concentrations were greater in females than in males, which is consistent with previous findings.[54,71] We did not observe a significant increase in circulating allopregnanolone concentrations 30 minutes after stress exposure in either sex, in contrast to previous studies in male rats.[11,52] The reason for this is not clear; however, the different duration/intensity of the swim stress used (2 minutes in the present study versus 10 minutes) may contribute. Moreover, allopregnanolone concentrations are known to peak 30-60 minutes later in the plasma than in the brain.[11] Nevertheless, following stress, allopregnanolone concentrations were greater in the frontal cortex, amygdala and brainstem of females compared to controls, as well as in the frontal cortex of males, consistent with previous findings.[11,52] Sex differences in central allopregnanolone concentrations may result from greater concentrations of the precursors, progesterone and DHP and/or sex differences in the activity of the converting enzymes in the brain. The strong positive correlation between central and peripheral progesterone concentrations, coupled with a lack of correlation between circulating and central allopregnanolone, and its precursor DHP, suggests that DHP and allopregnanolone are synthesised in the brain from progesterone following stress; however, it would be necessary to measure the activity of the converting enzymes in the brain to confirm this. Caruso et al[54] report a positive correlation between progesterone concentrations in the plasma and cerebral cortex in rats, which is consistent with our findings; however, in contrast to the present study, they also observed a direct correlation between pregnenolone, DHP and allopregnanolone levels in the

plasma and cerebral cortex. The reason for this difference is unclear; however, it is important to note that the correlations performed in the present study included data from both stressed and nonstressed rats within the same sex, whereas, in the previous study, correlations were analysed only under basal conditions with data from male and female rats combined. Thus, it is likely that the lack of correlation reported in the present study is a result of stress increasing central levels of pregnenolone, DHP and allopregnanolone, without affecting circulating levels.

It is not clear whether the sex differences in neurosteroids reported in the present study are of functional importance with respect to HPA axis regulation and further studies are needed to establish whether other stressors result in similar effects. Unlike the brainstem, the prefrontal cortex, amygdala and hippocampus do not innervate the PVN directly; rather, they modulate activity of the HPA axis via neural projections to GABAergic relay sites, such as the peri-PVN, the bed nucleus of the stria terminalis and the medial preoptic area, which exert considerable inhibitory tone over CRH neurones.[24,36] Thus, it is conceivable that sex differences in brain concentrations of neurosteroids with $GABA_A$ receptor agonist activity may lead to differential modulation of upstream inhibitory signalling pathways, resulting, for example, in a greater disinhibition of PVN CRH neurones in females, which could contribute to sex differences in HPA axis responses to stress.

As well as the increase in downstream metabolites of progesterone and DOC, the principal precursor for these steroid hormones, pregnenolone, was also increased throughout the brain following stress, and to a greater extent in the brains of females. This may indicate that, in females, either p450scc is more highly expressed/more active or that pregnenolone is metabolised at a different rate in females; however, this requires further study. Consistent with previous studies, we found no stress-induced change in plasma pregnenolone concentrations.[52]

One point to note is that the females used in the present study were randomly cycling, which may be expected to influence the steroid concentrations in the brain. However, there is very little variation in brain concentrations of pregnenolone, progesterone, DHP or allopregnanolone across the oestrous cycle when these are measured in the morning (as they were in the present study); indeed, circadian fluctuations in these steroids are far greater than those relating to any stage of the oestrous cycle.[84] Moreover, oestrous cycle stage does not affect basal corticosterone secretion[85] or the corticosterone response to swim stress[86] when sampling is performed in the morning.

The mechanisms involved in stress-induced increases in neuroactive steroid levels in the brain have yet to be established. One study has indicated that CRH increases concentrations of progesterone, DHP and allopregnanolone in the brain.[87] Steroidogenic enzymes such as 5α-reductase are known to be regulated by androgens[88,89] and, although adrenal-derived steroids have been shown to increase 3α-HSD expression in the brain in both sexes, gonadal steroids regulate central 3α-HSD expression in a sex-specific manner.[90] Given that testosterone was unaltered by stress, it is unlikely that it contributes to the rapid changes in stress-induced neuroactive steroids seen in the present study; however, testosterone may underlie sex differences in steroidogenic enzyme expression in the brain, and therefore influence the brain's capacity to produce neurosteroids.

With respect to rapid regulation of neurosteroid production, GABA may play a role via an ultrashort feedback loop. Brain regions that express neurosteroidogenic enzymes are densely innervated by GABAergic neurones and express abundant $GABA_A$ receptors.[91-93] Moreover, GABA or $GABA_A$ receptor agonists inhibit the de novo synthesis of neurosteroids[94] and neurosteroidogenic neurones are evidently under tonic inhibition by GABA.[94] Given that some neuroactive steroids, such as allopregnanolone, are potent allosteric modulators of $GABA_A$ receptor function,[19-21] it is conceivable that neurosteroids may control their own production by regulating the activity of $GABA_A$ receptors. Whether such a mechanism contributes to the sex differences in central neurosteroid concentrations requires further study; however, GABA receptor subunit expression does differ between males and females, under basal conditions and following stress.[95]

In summary, the data obtained in the present study demonstrate robust sex differences in the steroid response to acute swim stress and indicate sex-specific expression of steroidogenic enzymes in the brain. Taken together, our data also support a role for the de novo synthesis of neurosteroids by the brain following stress, particularly those derived from 5α-reductase activity (ie, DHDOC, DHP, allopregnanolone). The neurosteroid response to stress is likely to be adaptive and disparate neurosteroidogenesis may contribute to sex differences in HPA axis function; however, it is unclear whether greater neuroactive steroid levels in females confer an overall advantage or disadvantage. Stress-induced increases in neuroactive steroids are likely to have important neuroendocrine and/or behavioural effects to aid stress adaptation. For example, in addition to modulating HPA axis activity, allopregnanolone is known to have anxiolytic and analgesic actions.[82,96] Sex differences in the brain's response to stress may account for differences in the propensity to develop stress-related disorders between males and females, further highlighting the importance of considering sex when developing therapies.

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CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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SUPPORTING INFORMATION

Sex-dependent changes in neuroactive steroid concentrations in the rat brain following acute swim stress

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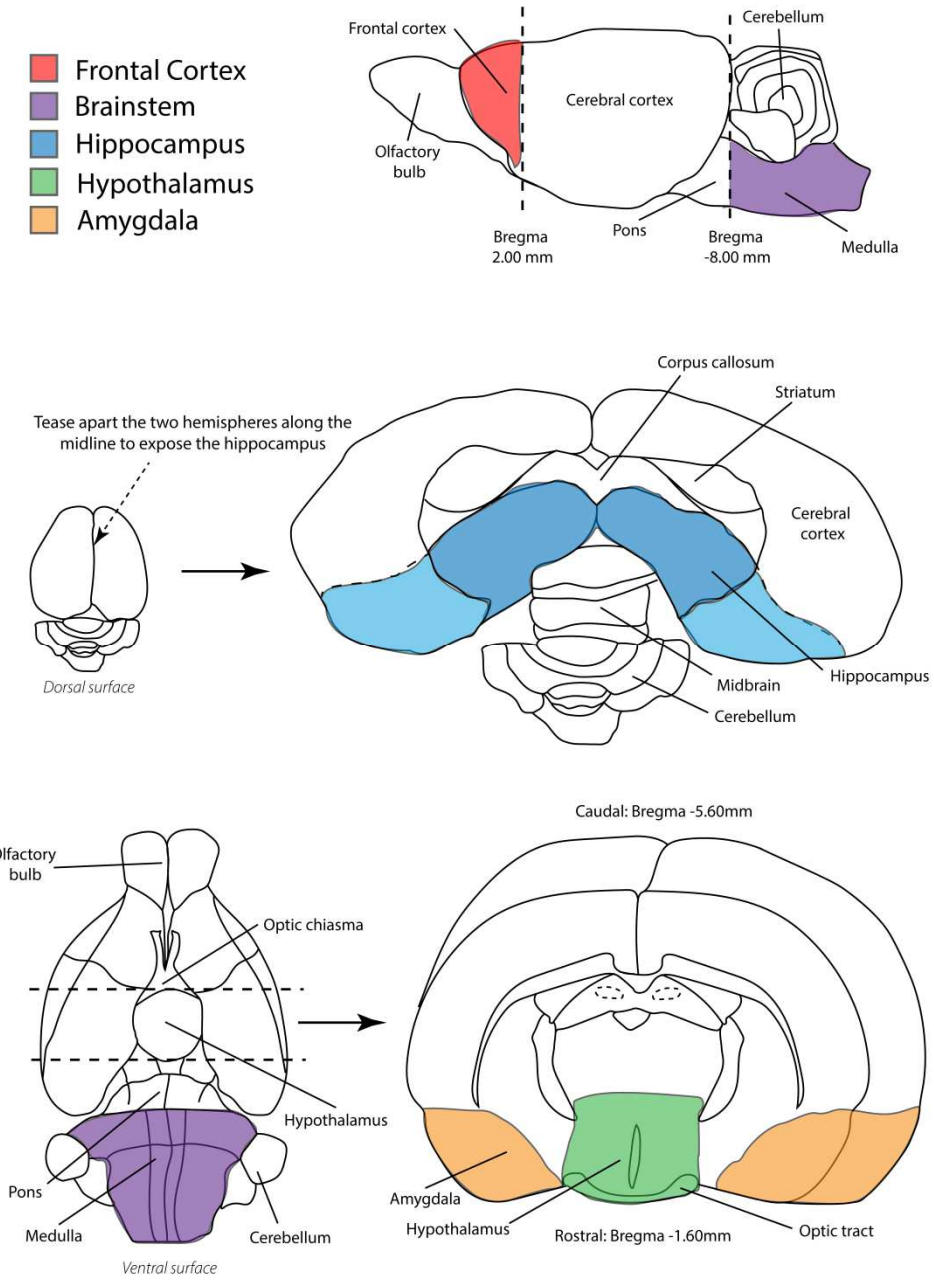
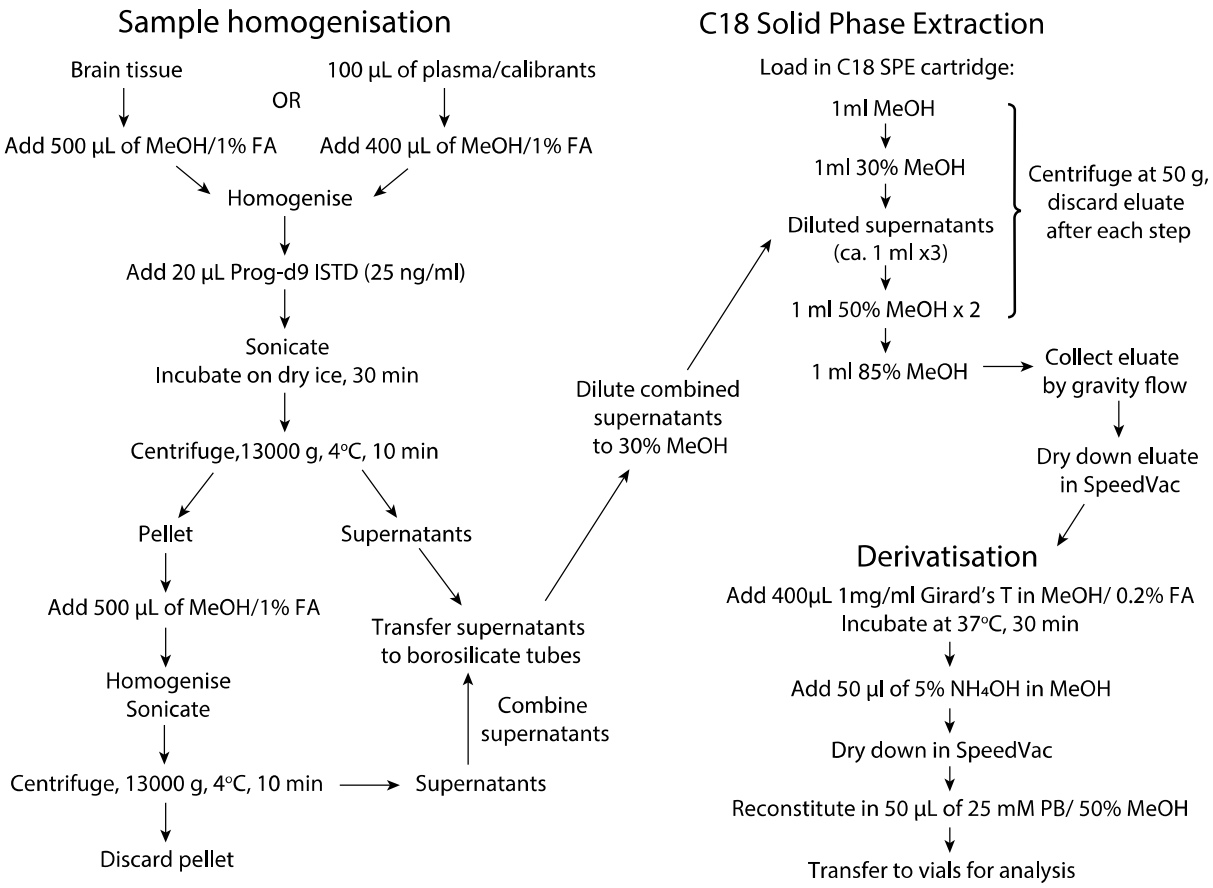
Supplementary Figure 1



Supplementary Figure 1: Schematic illustrating landmarks used for gross dissection of the different brain regions of interest.

Supplementary Figure 2



Supplementary Figure 2: Workflow for sample processing.

Brain and plasma samples were processed as above (*left column*), before solid phase extraction and derivatisation with Girard's T (*right column*).

Supplementary Table 1

LC parameters	
Injection volume	5µl; Partial loop injection
Flow rate	0.2 ml/min
Column temperature	40^{o}C
Injection needle rinse	20% Methanol
Gradient:	
-4 min to 0 min	5% B
0 min	Sample injection
0 min to 1 min	5% B to 20% B
1 min to 6 min	45% B to 50% B
6 min to 11 min	50% B
11 min to 12 min	50% B to 100% B
12 min to 14 min	100% B
14 min to 15 min	100% to 5% B
Divert to waste	Before 2 min, after 12 min
Total run time	19 min
MS parameters	
Electrospray ionisation mode	Positive
Capillary current	-4500V
Nebulizer gas	Nitrogen; 16 psi
Dry gas	8 L/min
Dry temperature	150^{o}C

Supplementary Table 1: Instrumentation set-up for liquid chromatography (*top panel*) and mass spectrometry (*bottom panel*)

Supplementary Table 2

Analyte	Molecular weight	Precursor ion (m/z)	Fragment ion (m/z)	Retention time (min)	Segment	Amplitude; Cut-Off
Corticosterone	346.5	460.2	401.1	3.5	1	117; 0.80
Testosterone	288.4	402.2	343.1	4.4	1	117; 0.80
DOC	330.5	444.2	385.1	4.6	1	120; 0.95
DHDOC	332.5	446.2	387.1	6.1	2	116; 0.80
Pregnenolone	316.5	430.2	371.1	6.3	2	116; 0.75
Progesterone-d9	323.5	437.5	368.3	8.2	3	116; 0.70
Progesterone	314.5	428.2	369.1	8.4	3	116; 0.75
DHP	316.5	430.2	371.1	8.6	3	116; 0.75
Allopregnanolone	318.5	432.2	373.1	10.4	4	116; 0.70

Supplementary Table 2: **Analytical parameters**

Analytical parameters for the target compounds and the internal standard (progesterone-d9). Derivatisation with Girard's T reagent produced a precursor ion $[M + G.T.]^+$, resulting in an addition of 114 Da to its absolute mass. Upon fragmentation by collision-induced fragmentation, a product ion with neutral loss of 59.1 Da (corresponding to the loss of the trimethylamine moiety) was produced, which was used for identification and quantification. Detection was carried out via multiple reaction monitoring, where no more than 3 analytes were monitored for each segment. Amplitude and cut-off parameters were optimised beforehand using a direct infusion method with a syringe pump.

Supplementary Table 3

Analyte	r^2	LOQ (pg/ml)	LOQ (pg, on column)	Calibration range (pg/ml)
Corticosterone	0.998	41	0.41	41 - 10000
DOC	0.998	41	0.41	41 - 10000
DHDOC	0.997	102.4	1.02	102.4 - 10000
Progesterone	0.998	256	2.56	256 - 25000
DHP	0.998	102.4	1.02	102.4 - 10000
Pregnenolone	0.998	102.4	1.02	102.4 - 10000
Allopregnanolone	0.996	256	2.56	256 - 25000
Testosterone	0.997	41	0.41	41 - 10000

Supplementary Table 3: Details of calibration curves

Calibration curves were constructed using the ratio of peak area of the target analyte and the peak area of progesterone-d9. Correlation coefficients (r^2) were calculated by weighted (1/x) regression analysis, obtained from the means of 6 calibration curves. The lower limit of quantification (LOQ) was defined as the concentration on the standard curve with a peak that is identifiable, discrete with a relative standard deviation of less than 20% and at least five times the signal of the blank. As 100µL of calibrant was used for sample processing, and only 5µL of the final reconstituted sample (50µL) was injected, the LOQ on-column (in pg) are also presented.

Supplementary Table 4

Analyte	QC level	Conc (pg/ml)	Recovery (%)	Intra-assay variability (%)	Inter-assay variability (%)	Accuracy (%)
Corticosterone	Low	500	74	16.9	24.9	111
	Medium	2000	71	19.5	22.9	104
	High	8000	84	14.4	22.0	98
DOC	Low	500	78	13.1	16.3	111
	Medium	2000	72	16.8	25.5	101
	High	8000	92	16.3	10.0	99
DHDOC	Low	500	95	16.5	15.1	140
	Medium	2000	82	6.6	24.9	113
	High	8000	108	9.0	7.8	112
Progesterone	Low	1250	71	17.9	13.7	126
	Medium	5000	80	5.5	6.5	98
	High	20000	83	8.0	4.1	104
DHP	Low	500	73	13.0	9.3	136
	Medium	2000	70	10.8	14.7	103
	High	8000	82	8.3	9.5	97
Pregnenolone	Low	500	88	17.6	24.8	110
	Medium	2000	81	8.4	16.6	89
	High	8000	95	12.5	9.6	93
Allopregnanolone	Low	1250	88	17.1	20.6	124
	Medium	5000	87	6.3	18.8	102
	High	20000	94	12.2	16.8	103
Testosterone	Low	500	95	18.5	16.5	132
	Medium	2000	105	7.7	8.8	106
	High	8000	106	10.4	10.3	109

Supplementary Table 4: **Assay performance characteristics**

Assay precision was examined using low, medium or high concentration (conc) of quality control standards (QC low, QC medium, QC high) in 4% BSA. 100 µl of QC standards were used and were processed as described in the methods, along with a set of standard calibrants. Intra-assay variability referred to the relative standard deviation (% RSD) of the values (pg/ml) obtained from four independent samples in a single run, against a single calibration curve. Inter-assay variability referred to the % RSD of four independent samples across four different runs, against four different calibration curves. Recovery was determined by comparing the peak areas for QC standards spiked before and after solid phase extraction (n=3). Accuracy was calculated using the following formula: '(obtained concentration/known concentration) x 100%', using the means of four independent samples. All samples were injected in duplicate and the means were used for all calculations.

Appendix C: Additional comparisons of steroid concentrations between sexes and between plasma and brain regions

Appendix C1: Three-way ANOVA analysing effects of acute stress X prenatal stress X sex on neuroactive steroid concentrations (Chapter 4)

Three-way ANOVAs were carried out in R-studio using natural log transformed data (except for corticosterone), with sex, acute stress and prenatal stress as the main factors investigated. There were no main effects of prenatal stress observed in any of the analytes in any of the regions, and there were also no three-way interactions in any of the analytes observed. Two-way interactions were present, especially for progesterone, DHP and allopregnanolone. This was mostly due to the differential response in progesterone and its metabolites following swim stress in the female offspring, discussed in section 4.4.4. Main effects of acute stress were observed for all analytes except for testosterone. Robust main effects of sex were also observed for almost all analytes in all tissues, indicating differences in absolute concentrations of steroids.

Corticosterone	**Main effects**			**Interactions**
	Sex	Acute Stress	Prenatal Stress	
Plasma	ns	$F_{1,70}$=49.3, ***	ns	ns
Frontal Cortex	$F_{1,70}$=5.43, *	$F_{1,70}$=44.8, ***	ns	ns
Hypothalamus	$F_{1,70}$=10.7, **	$F_{1,70}$=49.7, ***	ns	ns
Hippocampus	ns	$F_{1,70}$=87.3, ***	ns	Prenatal Stress x Sex, $F_{1,70}$=5.93, *
Amygdala	ns	$F_{1,70}$=109.8, ***	ns	ns
Brainstem	$F_{1,70}$=14.5, ***	$F_{1,70}$=37.9, ***	ns	ns

DOC (log transformed)	**Main effects**			**Interactions**
	Sex	Acute Stress	Prenatal Stress	
Plasma	$F_{1,70}$=10.5, **	$F_{1,70}$=74.3, ***	ns	Acute Stress x Sex, $F_{1,70}$=4.37, *
Frontal Cortex	$F_{1,70}$=14.8, ***	$F_{1,70}$=78.0, ***	ns	ns
Hypothalamus	$F_{1,70}$=60.5, ***	$F_{1,70}$=55.4, ***	ns	ns
Hippocampus	$F_{1,70}$=102.6, ***	$F_{1,70}$=21.4, ***	ns	ns
Amygdala	$F_{1,70}$=20.1, ***	$F_{1,70}$=100.6, ***	ns	ns
Brainstem	$F_{1,70}$=36.4, ***	$F_{1,70}$=64.6, ***	ns	ns

DHDOC (log transformed)	**Main effects**			**Interactions**
	Sex	Acute Stress	Prenatal Stress	
Plasma	$F_{1,69}$=219, ***	ns	ns	ns
Frontal Cortex	$F_{1,69}$=119.9, ***	$F_{1,69}$=39.1, ***	ns	ns
Hypothalamus	ns	$F_{1,69}$=29.1, ***	ns	ns
Hippocampus	$F_{1,69}$=76.3, ***	$F_{1,69}$=65.0, ***	ns	ns
Amygdala	$F_{1,69}$=61.3, ***	$F_{1,69}$=41,3. ***	ns	ns
Brainstem	$F_{1,69}$=81.6, ***	$F_{1,69}$=42.3, ***	ns	ns

THDOC (log transformed)	**Main effects**			**Interactions**
	Sex	Acute Stress	Prenatal Stress	
Plasma	$F_{1,69}$=52.4, ***	$F_{1,69}$=15.2, ***	ns	Acute Stress x Sex, $F_{1,69}$=6.67, *
Frontal Cortex	$F_{1,69}$=224.0, ***	$F_{1,69}$=26.6, ***	ns	ns
Hypothalamus	ns	$F_{1,69}$=29.9, ***	ns	ns
Hippocampus	$F_{1,69}$=57.2, ***	$F_{1,69}$=16.2, ***	ns	ns
Amygdala	$F_{1,69}$=32.6, ***	$F_{1,69}$=19.7, ***	ns	ns
Brainstem	$F_{1,69}$=48.2, ***	$F_{1,69}$=28.3,***	ns	ns

Progesterone (log transformed)	Main effects			Interactions
	Sex	Acute Stress	Prenatal Stress	
Plasma	$F_{1,70}$=161.7, ***	$F_{1,70}$=27.5, ***	ns	Acute Stress x Sex, $F_{1,70}$=2.84, p=0.09
Frontal Cortex	$F_{1,70}$=224.0, ***	$F_{1,70}$=43.6, ***	ns	Acute Stress x Sex, $F_{1,70}$=4.92, *
Hypothalamus	$F_{1,70}$=73.5, ***	$F_{1,70}$=37.9, ***	ns	Acute Stress x Sex, $F_{1,70}$=8.12, **
Hippocampus	$F_{1,70}$=194.9, ***	$F_{1,70}$=44.1, ***	ns	Acute Stress x Sex, $F_{1,70}$=15.0, ***
Amygdala	$F_{1,70}$=198.1, ***	$F_{1,70}$=39.5, ***	ns	Acute Stress x Sex, $F_{1,70}$=5.04, *
Brainstem	$F_{1,70}$=239.1, ***	$F_{1,70}$=52.7, ***	ns	Acute Stress x Sex, $F_{1,70}$=10.2, **

DHP (log transformed)	Main effects			Interactions
	Sex	Acute Stress	Prenatal Stress	
Plasma	$F_{1,70}$=99.3, ***	ns	ns	ns
Frontal Cortex	$F_{1,70}$=311.,8, ***	$F_{1,70}$=20.1, ***	ns	ns
Hypothalamus	$F_{1,70}$=53.5, ***	$F_{1,70}$=17.1, ***	ns	Acute Stress x Sex, $F_{1,70}$=5.54, *
Hippocampus	$F_{1,70}$=229.7, ***	$F_{1,70}$=8.53, **	ns	Acute Stress x Sex, $F_{1,70}$=4.74, *
Amygdala	$F_{1,70}$=178.9, ***	$F_{1,70}$=11.5, **	ns	Acute Stress x Sex, $F_{1,70}$=4.78, *
Brainstem	$F_{1,70}$=250.2, ***	$F_{1,70}$=33.1, ***	ns	Acute Stress x Sex, $F_{1,70}$=10.3, **

Allopregnanolone (log transformed)	Main effects			Interactions
	Sex	Acute Stress	Prenatal Stress	
Plasma	$F_{1,70}$=647.6, ***	$F_{1,70}$=4.1, ***	ns	Acute Stress x Sex, $F_{1,70}$=6.5, *
Frontal Cortex	$F_{1,70}$=297.,1, ***	$F_{1,70}$=20.3, ***	ns	ns
Hypothalamus	$F_{1,70}$=338.8, ***	$F_{1,70}$=28.3, ***	ns	Acute Stress x Sex, $F_{1,70}$=4.94, *
Hippocampus	$F_{1,70}$=335.7, ***	$F_{1,70}$=14.8, ***	ns	Acute Stress x Sex, $F_{1,70}$=10.4, **
Amygdala	$F_{1,70}$=230.7, ***	$F_{1,70}$=13.3, ***	ns	ns
Brainstem	$F_{1,70}$=374.1, ***	$F_{1,70}$=38.8, ***	ns	Acute Stress x Sex, $F_{1,70}$=12.8, ***

Pregnenolone (log transformed)	Main effects			Interactions
	Sex	Acute Stress	Prenatal Stress	
Plasma	$F_{1,70}$=407.8,***	$F_{1,70}$=59.0, ***	ns	ns
Frontal Cortex	$F_{1,70}$=103.9, ***	$F_{1,70}$=111.4, ***	ns	ns
Hypothalamus	$F_{1,70}$=87.3, ***	$F_{1,70}$=95.8	ns	ns
Hippocampus	$F_{1,70}$=123.8, ***	$F_{1,70}$=121.0, ***	ns	ns
Amygdala	$F_{1,70}$=123.0, ***	$F_{1,70}$=185.9, ***	ns	ns
Brainstem	$F_{1,70}$=234.0,***	$F_{1,70}$=99.3, ***	ns	ns

Testosterone (log transformed)	Main effects			Interactions
	Sex	Acute Stress	Prenatal Stress	
Plasma	$F_{1,71}$=499.1, ***	ns	ns	ns
Frontal Cortex	$F_{1,71}$=430.7, ***	ns	ns	ns
Hypothalamus	$F_{1,71}$=153.7, ***	ns	ns	ns
Hippocampus	$F_{1,71}$=447.3, **	ns	ns	ns
Amygdala	$F_{1,71}$=426.0, ***	ns	ns	ns
Brainstem	$F_{1,71}$=133.6, ***	ns	ns	ns

Table C1: Results of three-way ANOVA for analysing the effects of acute stress X prenatal stress X sex on neuroactive steroid concentrations. * p<0.05, ** p<0.01, *** p<0.001. Data (except for corticosterone) was log transformed before carrying out three-way ANOVA (Appendix A)

Appendix C2: Two-way ANOVA for analysing differences in concentrations of neuroactive steroids between plasma and the brain (Chapter 4)

In order to determine if there are differences between concentrations of steroids between plasma and brain regions, a two-way ANOVA was carried out, where region (plasma and the respective brain regions) and swimming stress were analysed as the main factors. Swimming stress was added as a main factor as it was shown to affect steroid concentrations dramatically (section 4.3, results section). However, as steroid concentrations between controls and PNS were generally not different (section 4.3), control and PNS groups were collapsed within each swimming stress group. Males and females were analysed separately in this case. F-values for main effect of region and interactions are presented in Table C2. Post-hoc pairwise comparisons were carried out using Student-Newman-Keuls test, and only results from the comparison between plasma and the various brain regions are presented in Table C3. Brain regions which had significantly different steroid concentrations as compared to plasma ($P<0.05$), either basally or upon acute stress, are presented (Table C3), with the direction of difference also reported.

Two-way ANOVA	Male		Female	
	Main effect of region	Region X Swim stress interaction	Main effect of region	Region X Swim stress interaction
Corticosterone	$F_{5,216}$ = 57.7, ***	$F_{5,216}$ = 11.6, ***	$F_{5,216}$ = 36.1, ***	$F_{5,216}$ = 13.2, ***
DOC	$F_{5,216}$ = 1.2, n.s.	$F_{5,216}$ = 1.08, n.s.	$F_{5,216}$ = 5.03, **	$F_{5,216}$ = 1.3, n.s.
DHDOC	$F_{5,216}$ = 24.5, ***	$F_{5,216}$ = 6.9, ***	$F_{5,216}$ = 45.2, ***	$F_{5,216}$ = 10.9, ***
THDOC	$F_{5,216}$ = 17.3, ***	$F_{5,216}$ = 4.14, **	$F_{5,216}$ = 7.7, ***	$F_{5,216}$ = 2.46, **
Progesterone	$F_{5,216}$ = 25.2, ***	$F_{5,216}$ = 9.9, ***	$F_{5,216}$ = 4.7, **	$F_{5,216}$ = 0.54, n.s.
DHP	$F_{5,216}$ = 25.7, ***	$F_{5,216}$ = 12.3, ***	$F_{5,216}$ = 28.6, ***	$F_{5,216}$ = 1.2, n.s.
Allopregnanolone	$F_{5,216}$ = 7.85, ***	$F_{5,216}$ = 3.4, **	$F_{5,216}$ = 6.5, ***	$F_{5,216}$ = 0.5, n.s.
Pregnenolone	$F_{5,216}$ = 13.7, ***	$F_{5,216}$ = 4.9, ***	$F_{5,216}$ = 19.5, ***	$F_{5,216}$ = 4.1, **
Testosterone	$F_{5,216}$ = 9.4, ***	$F_{5,216}$ = 0.25, n.s.	$F_{5,216}$ = 48.5,***	$F_{5,216}$ = 0.35, n.s.

Table C2: Results of two-way ANOVA for analysing differences in concentrations of neuroactive steroids between plasma and brain regions.
Two-way ANOVA was carried out, with swimming stress and region (plasma and the respective brain regions) as the main factors. ** $P<0.01$, ***$P<0.001$, n.s. not significant

(A) Male	Amygdala		Brainstem		Frontal Cortex		Hippocampus		Hypothalamus	
	Basal	Swim	Basal	Swim	Basal	Swim	Basal	Swim	Basal	Swim
Corticosterone	L	L	L	L	L	L	L	L	L	L
DOC	-	-	-	-	-	-	-	-	-	-
DHDOC	-	-	-	H	-	-	-	H	H	H
THDOC	-	-	-	H	-	-	-	-	H	H
Progesterone	-	-	-	H	-	-	-	H	H	H
DHP	-	-	-	H	-	-	-	H	H	H
Allopregnanolone	-	H	-	H	-	H	-	H	H	H
Pregnenolone	-	H	-	H	-	H	-	H	H	H
Testosterone	-	-	-	-	-	-	H	H	-	-

(B) Female	Amygdala		Brainstem		Frontal Cortex		Hippocampus		Hypothalamus	
	Basal	Swim	Basal	Swim	Basal	Swim	Basal	Swim	Basal	Swim
Corticosterone	L	L	L	L	L	L	L	L	L	L
DOC	-	-	-	-	-	-	-	-	-	H
DHDOC	-	-	H	H	-	-	-	H	-	H
THDOC	-	-	H	H	-	-	-	-	-	H
Progesterone	-	-	H	H	-	H	-	-	-	H
DHP	-	-	H	H	-	-	-	-	-	H
Allopregnanolone	L	L	-	-	L	L	L	L	L	L
Pregnenolone	-	H	H	H	-	H	-	H	-	H
Testosterone	-	-	H	H	-	-	-	-	H	H

Table C3: Results of post-hoc SNK test for analysing differences in concentrations of neuroactive steroids between plasma and brain regions. Post-hoc pairwise comparisons using Student-Newman-Keuls test comparing plasma steroid concentrations with steroid concentrations in each brain region are presented for males in panel (A) and females in panel (B). H: higher in the brain region as compared to plasma, L: lower in the brain region as compared to plasma, - : no difference in concentrations between plasma and brain regions. In general, corticosterone concentrations were always lower in the brain as compared to the plasma. For allopregnanolone, concentrations were always lower in the female brain as compared to female plasma. In males however, allopregnanolone concentrations were greater in the brain as compared to the plasma, especially in acute stress conditions. With the exception of DOC, where concentrations were similar in the plasma and brain, for other steroids, concentrations were generally greater in the brain regions as compared to the plasma, although for certain brain regions, concentrations were not different between plasma and the brain

Appendix D: Changes in receptor expression and neuronal markers in juvenile offspring brain, graphs and statistical analyses (Chapter 6)

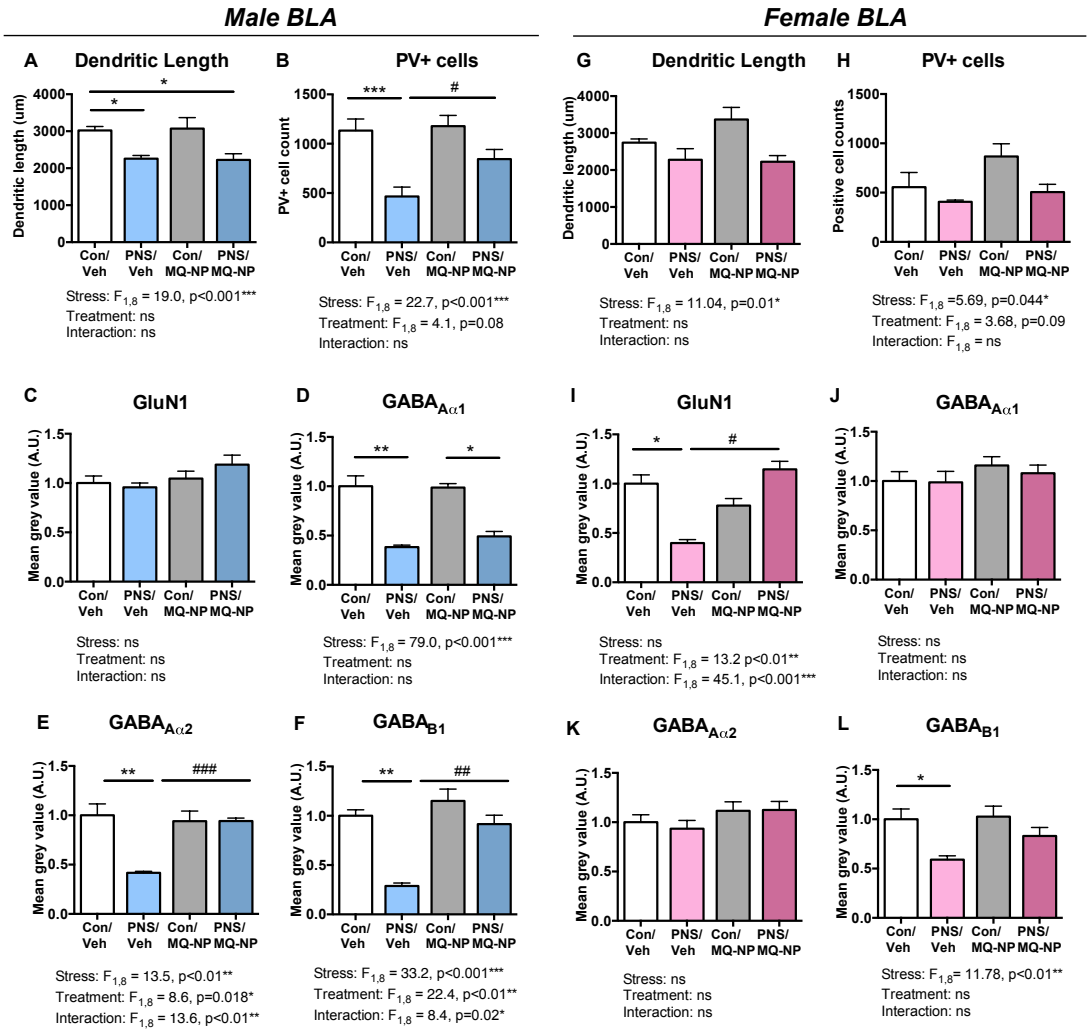


Figure D1: Effects of prenatal stress and maternal MitoQ-NP administration on neurobiological markers in the juvenile offspring basolateral amygdala. Two way ANOVA results are reported under the graphs while results for Bonferroni-corrected pairwise comparisons are annotated on the graphs. Asterisks represent differences between control and PNS groups (where * p<0.05, ** p<0.01, *** p<0.001), while hashes represent differences between vehicle and MitoQ-NP treated groups (where # p<0.05, ## p<0.01, ### p<0.001). Con: Control, PNS: Prenatal stress, Veh: Maternal vehicle treatment, MQ-NP: Maternal MitoQ-NP treatment, ns: not significant. n=3 rats per group. Data was analysed from 30 sampling views per rat by Dr Tom J Phillips.

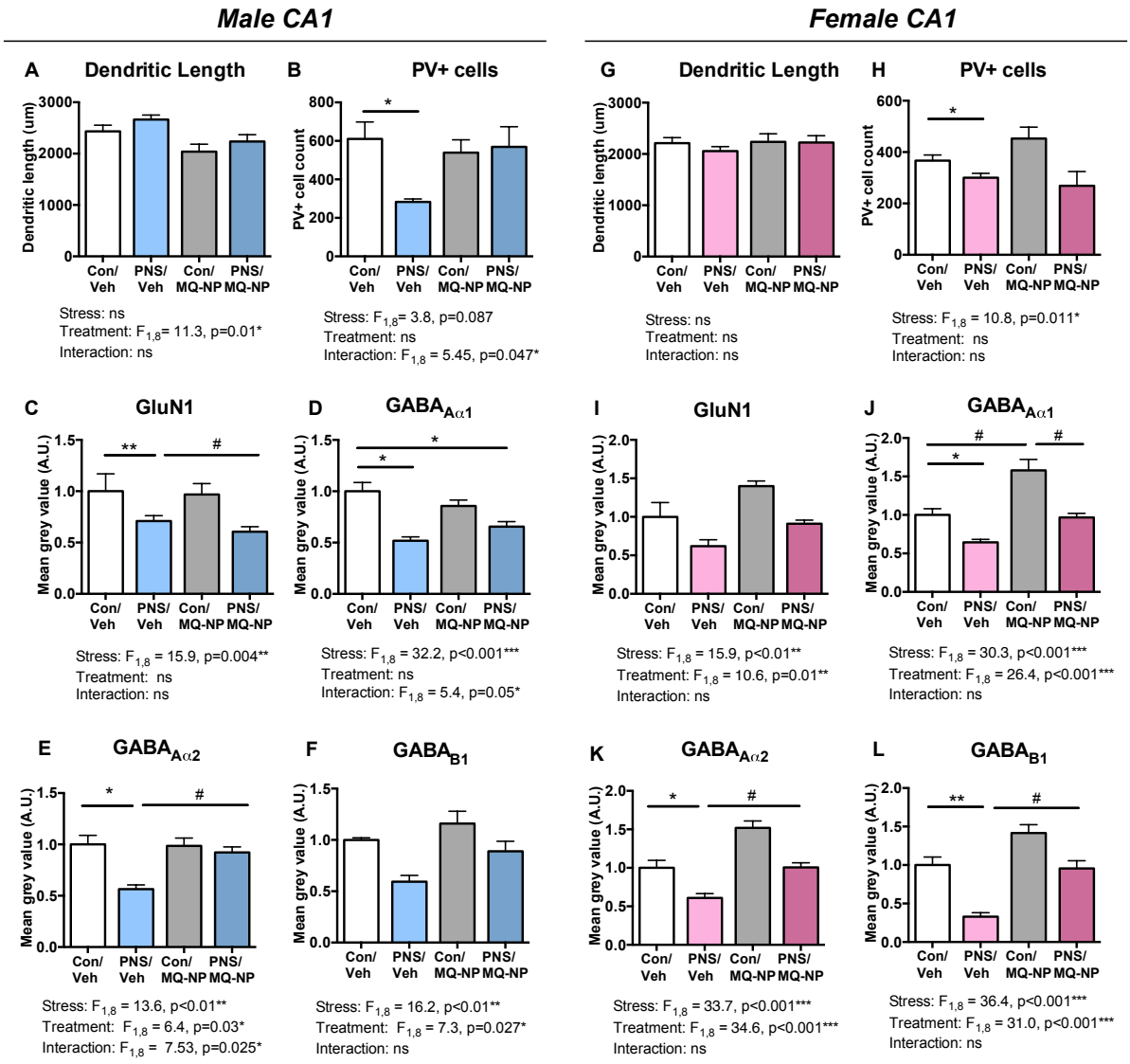


Figure D2: Effects of prenatal stress and maternal MitoQ-NP administration on neurobiological markers in the hippocampal CA1 region of the juvenile offspring. Two way ANOVA results are reported under the graphs while results for Bonferroni-corrected pairwise comparisons are annotated on the graphs. Asterisks represent differences between control and PNS groups (where * p<0.05, ** p<0.01), while hashes represent differences between vehicle and MitoQ-NP treated groups (where # p<0.05). Con: Control, PNS: Prenatal stress, Veh: Maternal vehicle treatment, MQ-NP: Maternal MitoQ-NP treatment, ns: not significant. n=3 rats per group. Data was analysed from 30 sampling views per rat by Dr Tom J Phillips.

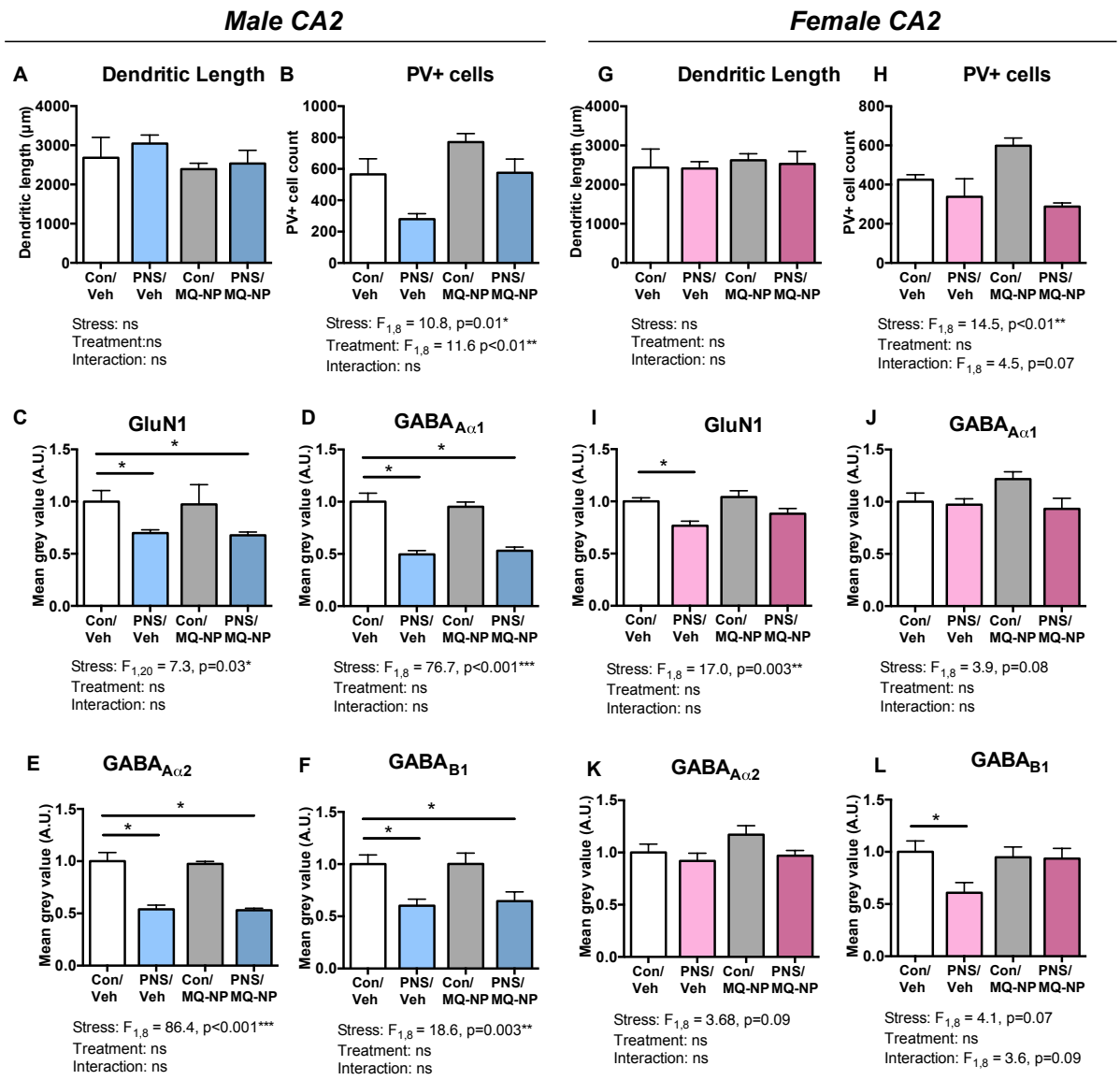


Figure D3: Effects of prenatal stress and maternal MitoQ-NP administration on neurobiological markers in the hippocampal CA2 region of the juvenile offspring. Two way ANOVA results are reported under the graphs while results for Bonferroni-corrected pairwise comparisons are annotated on the graphs. Asterisks represent differences between control and PNS groups (where * p<0.05,). Con: Control, PNS: Prenatal stress, Veh: Maternal vehicle treatment, MQ-NP: Maternal MitoQ-NP treatment, ns: not significant. n=3 rats per group. Data was analysed from 30 sampling views per rat by Dr Tom J Phillips.

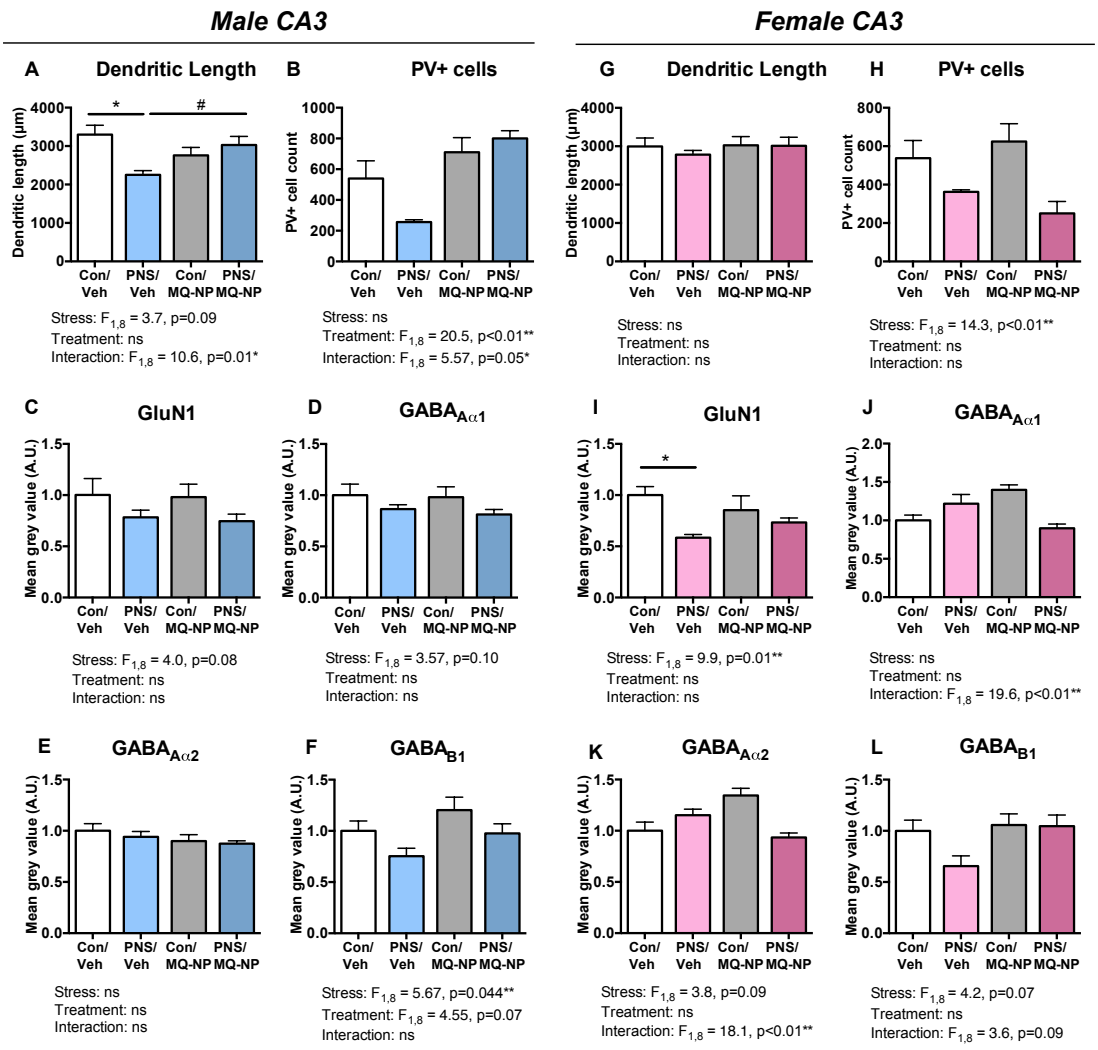


Figure D4: Effects of prenatal stress and maternal MitoQ-NP administration on neurobiological markers in the hippocampal CA3 region of the juvenile offspring. Asterisks represent differences between control and PNS groups (where * p<0.05), while hashes represent differences between vehicle and MitoQ-NP treated groups (where # p<0.05). Con: Control, PNS: Prenatal stress, Veh: Maternal vehicle treatment, MQ-NP: Maternal MitoQ-NP treatment, ns: not significant. n=3 rats per group. Data was analysed from 30 sampling views per rat by Dr Tom J Phillips.

Appendix E: Changes in neuronal cultures exposed to placental-conditioned media and foetal plasma, graphs and statistical analyses (Chapter 6)

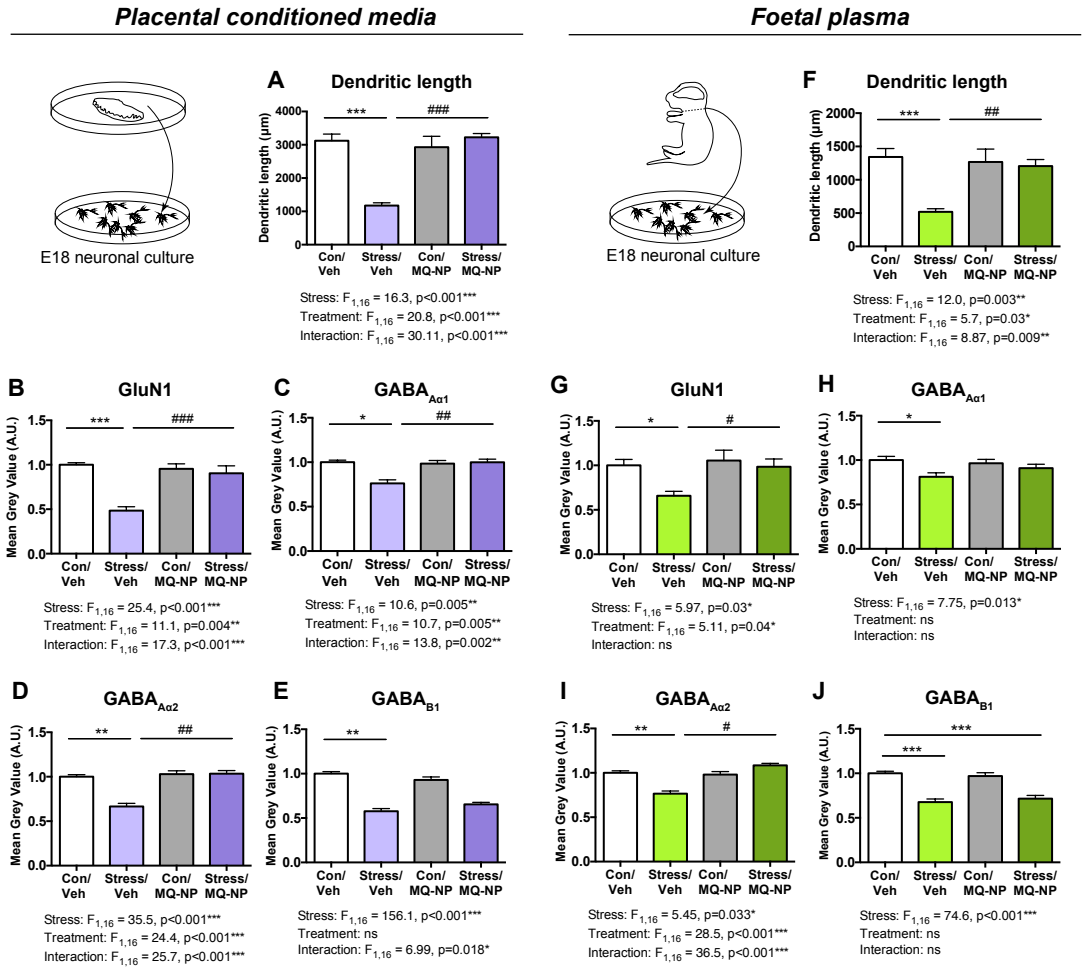


Figure E1: Effects of placental-conditioned media and foetal plasma on neuronal characteristics of E18 neuronal cultures. Placental-conditioned media and foetal plasma were collected then transported to the University of Bristol, where they were applied to neuronal cultures by Dr Tom J Phillips. Dendritic length (A, F), GluN1 expression (B, G); and GABA $_{Aα2}$ expression (D, I) was lower in neurones for which placental-conditioned media and foetal plasma from stressed animals was applied. In several cases, this effect was not observed when placental-conditioned media and foetal plasma from stressed/maternal MitoQ-NP treated groups was applied. A similar decrease in $GABA_{B1}$ was observed, but no normalisation was observed (E, J). For $GABA_{Aα1}$, a normalisation was observed in the placental-conditioned media (C) but not for foetal plasma (H). Asterisks represent differences between control and PNS groups (where * p<0.05, ** p<0.01, *** p<0.001), while hashes represent differences between vehicle and MitoQ-NP treated groups (where

# p<0.05, ## p<0.01, ### p<0.001). Con: Control, PNS: Prenatal stress, Veh: Maternal vehicle treatment, MQ-NP: Maternal MitoQ-NP treatment. n=5 per group.